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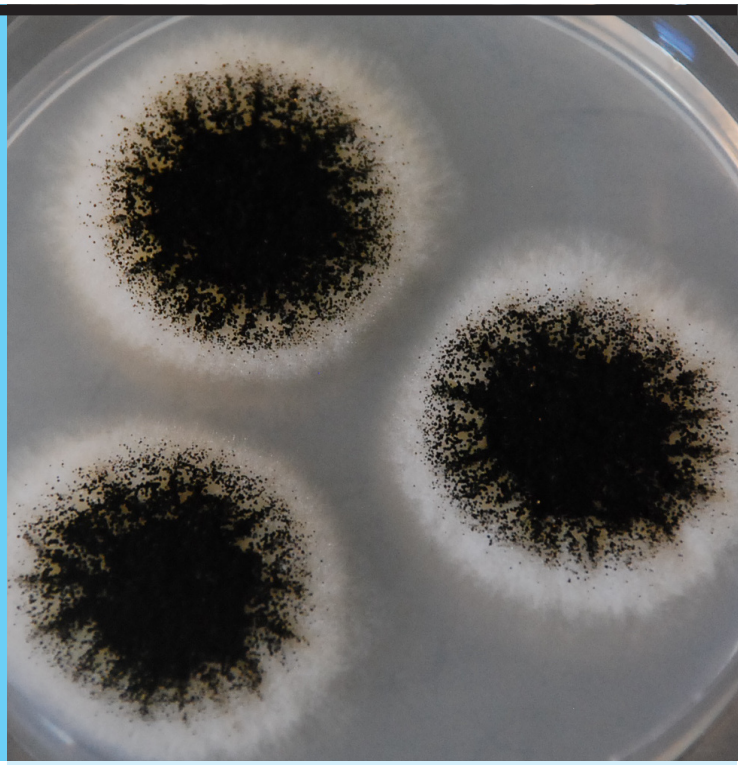
Significance and occurrence of fumonisins from *Aspergillus niger*



Jesper Mølgaard Mogensen
Ph.D. Thesis
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Center for Microbial Biotechnology
Department of Systems Biology

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Preface

This thesis is submitted to the Technical University of Denmark in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in chemistry. The work has been carried out between October 2008 and December 2011, at the Center for Microbial Biotechnology at the Department of Systems Biology under the main supervision of associate professor **Kristian Fog Nielsen** with associate professor **Thomas Ostenfeld Larsen** and Professor **Jens Christian Frisvad** as co-supervisors. The project was funded by the Danish Food Industry Agency (grant 3304-FVEP-07-730-01).

First and foremost I would like to thank my three supervisors for three exciting, interesting and productive years. You have all been very enthusiastic throughout the project and your inspiration scientific advice, constructive criticism and support have been valuable during the PhD study.

A thank also goes to **Anoop Kumar Sharma** for his help with the determination of the genotoxicity of fumonisin at DTU Food in Mørkhøj.

I had several opportunities to collaborate with national and international research groups, on various manuscripts. **Rudolf Krska**, **Michael Sulyok** and **Elisabeth Varga** from IFA-Tulln, Austria; **Gordon Shephard** and **Liana van der Westhuizen** from the PROMEC unit, South Africa; **Peter Have Rasmussen** from DTU-FOOD; **Robert Samson** and **Janos Varga** from CBS-KNAW, The Netherlands; **Roman Labuda** from Romer Labs, Austria; **Alena Kubátová** from Charles University, Czech Republic are all thanked for their collaboration and insightful discussions on the various manuscripts included in this thesis.

I would also like to thank **Hanne Jacobsen** and **Jesper Mogensen** for the help with the analytical instrumentation. **Tanja Thorskov Bladt**, **Marie Louise Klejnstrup** and **Lisette Knoth-Nielsen** are acknowledged for their efforts in purifying fumonisins from *A. niger* for the genotoxicity studies.

During my PhD project I have had the opportunity to work with several productive students. Most noteworthy are **Stine Mørchholdt Sørensen** for her work on the single kernel manuscript, as well as **Kirsten Amalie Møller** and **Pernille von Freiesleben** for their efforts with *Tolypocladium*.

My current and former office mates, **Maria Månsson**, **Mads Holger Rønnest**, **Peter Boldsen Knudsen** and **Tanja Thorskov Bladt** have all been invaluable over the years with your advice, positive attitude and great laughs. I would also like to thank the rest of my colleagues at CMB for contributing to a great work environment where you always feel welcome.

Lastly I would like to thank my family for their support the past three years. I am furthermore grateful for having a great wife and for her invaluable encouragement and support the last 3 years.

Thesis outline

The overall aim of this thesis was to determine whether fumonisins produced by *Aspergillus niger* is an overlooked health risk in Denmark and worldwide. The objectives were to survey the occurrence and levels of fumonisin in the relevant food commodities such as grapes **(paper 2)**, wine **(Paper 3)**, raisins **(Paper 2+4)**, black tea **(Paper 5),** and peanuts **(Paper 6)**; investigation of other fungi with similar genes for potential fumonisin production **(Paper 8)**; and to determine the genotoxic potential of fumonisins from *A. niger*. Other objectives were to investigate the effect of physiological parameters on the production of fumonisins by *A. niger* **(Paper 1)** and the fumonisin content in single maize kernels **(Paper 7)**. The secondary metabolism of black Aspergilli was reviewed in **(Paper 9).**

The first chapter introduces mycotoxins with special focus on black Aspergilli and the toxins they produce, specifically fumonisins. Chapter 2 outlines the experimental setup for the thesis. In chapter 3 the main results of the articles are presented and discussed. Finally, chapter 4 is the overall conclusion.

Summary

Fumonisins is a well-studied group of mycotoxins, mainly produced in maize by *Fusarium* species. However with the recent discovery of a fumonisin production by *Aspergillus niger*, other food commodities are at risk, since *A. niger* is a ubiquitous contaminant of many food and feed products.

The objective of this thesis was to determine the significance and occurrence of fumonisins from *Aspergillus niger* in food, the frequency of fumonisin production in *A. niger* isolates, as well as studies of the effect of physiological factors affecting fumonisin production. Major findings in this context have been the documentation of the production of fumonisins in raisins and peanuts, and occurrence of *A. niger* derived fumonisins in retail wine and raisins.

Physiological investigations have demonstrated that fumonisin production in *A. niger* occurs at temperatures between 20-37 °C. Three water activity lowering solutes, glycerol, NaCl, and sucrose were used. All *A. niger* strains responded differently on the three solutes. Addition of NaCl and sucrose increased the fumonisin production, where addition of glycerol decreased the production.

Fumonisin production in grape and derived products was investigated. Sixty-six *A. niger* strains were isolated and screened for a fumonisin production, and 77 % produced fumonisin on agar media. Worst-case scenarios were made by inoculation of *A. niger* on grapes and solidified the thought that a potential fumonisin production/contamination in grapes and derived products could occur. All tested *A. niger* strains produced fumonisins, not only when growing on grapes, but also on dried grapes and re-moistened raisins. By screening of 77 wine and 21 raisins samples, 18 wine (1-25 µg FB_2/l) and 10 raisin (1.3-13 µg FB_2/kg) samples contained detectable amounts of fumonisins.

An investigation of peanuts and peanut butter did not show any sign of fumonisin contamination, even though *A. niger* produced high amounts of fumonisins in worst case scenarios on shelled peanuts. Black and puerh tea were also examined, since *A. niger* were claimed to be the primary black Aspergilli in these. After isolation of 47 black Aspergilli, who all turned out to be *A. acidus* and did not produce neither ochratoxin or fumonisins, no significant risk from these toxins were associated with this intake.

Single kernel analysis of maize showed that fumonisins were present in 15% of the kernels at varying concentration. The frequency of highly contaminated kernels was 4 %. An estimated

calculation showed that the fumonisin content could be reduced with more than 70 % by sorting the maize. Other frequently found fungal metabolites in the maize kernels were chanoclavine, emodin, equisetin, and fusaric acid.

The biotechnologically important fungus *Tolypocladium*, a producer of the immunosuppressive drug cyclosporin, was identified as a new fumonisin producer. Three species within the genera produced fumonisin B_2 and B_4, and due to the widespread ability within the genus, it should be determined if this mycotoxin production occurs under industrial settings.

Dansk sammendrag

Fumonisiner er en gruppe velstuderede mykotoksiner, der hovedsagligt produceres af *Fusarium* i majs. Med opdagelsen af, at *Aspergillus niger* kan producere fumonisiner, er der flere grupper fødevarer som kan indeholde fumonisin, da *A. niger* er en hyppig kontaminant på mange forskellige fødevarer.

Formålet med denne Ph.D. afhandling var at undersøge udbredelsen og betydningen af *A. niger*'s produktion af fumonisiner i fødevarer, frekvensen af fumonisin producerende *A. niger*, samt effekten af fysiologiske faktorer på fumonisin produktionen. De væsentligste opdagelser, er fund af fumonisiner i indkøbte vine og rosiner, samt dokumentation af høj fumonisin produktion i kunstigt inficerede rosiner og jordnødder.

Fysiologiske undersøgelser viste, at *A. niger* producerer fumonisin ved temperaturer mellem 20-37 °C. Til at sænke vandaktiviteten blev NaCl, Glycerol og sucrose anvendt. Alle *A. niger* stammer reagerede forskelligt på de tre forskellige typer af medier med sænket vandaktivitet. Tilsætning af lave mængder NaCl og sucrose forøgede fumonisin produktionen, mens tilsætning af glycerol formindskede den.

Produktionen af fumonisiner i fødevarer herunder druer og afledte produkter blev også undersøgt. Seksogtres *A. niger* stammer blev isoleret fra rosiner og screenet for fumonisin produktion. I alt producerede 77 % af stammerne fumonisin på agar medier. *A. niger* blev podet på druer for at efterligne værst tænkelige scenarier. Da samtlige *A. niger* producerede fumonisin under disse forhold, var der mulighed for at fumonisin kunne findes i drue afledte produkter. Alle de testede stammer producerede desuden fumonisin i tørrede druer og fugtede rosiner. Syvoghalvfjerds vine og 21 rosin mærker blev undersøgt for fumonisiner og 18 vine (1-25 µg FB_2/l) samt 10 rosin mærker (1.3-13 µg FB_2/kg) indeholdt målbare mængder.

Undersøgelsen af fumonisiner i jordnødder og jordnøddesmør, påviste ikke tilstedeværelsen af disse mykotoksiner på trods af, at *A. niger* kan producere fumonisin i værst tænkelige scenarier i jordnødder. Sort og Puerh te blev også undersøgt, da det var påstået, at *A. niger* var den dominerende organisme fra section *Nigri*. Men efter at have isoleret 47 sorte aspergiller, som alle blev identificeret som *A. acidus*, der ikke producerede hverken ochratoxin A eller fumonisiner, var der ikke tegn på en signifikant risiko ved indtagelse af te.

Enkelt kerne analyse af majs påviste, at fumonisin fandtes i 15 % af kernerne med varierende koncentration. Frekvensen af kerner med en høj koncentration af fumonisin var 4 %. Hvis disse højt inficerede kerner kan frasorters vil fumonisinindholdet i majsen reduceres med 70 %. Øvrige hyppige svampe metabolitter detekteret i majs kernerne var chanoclavine, emodin, equisetin, samt fusaric acid.

Den bioteknologiske anvendte skimmelsvamp *Tolypocladium*, der anvendes til produktion af det immunosuppressive medikament cyclosporin, blev identificeret som en ny fumonisin producent. Tre arter i denne slægt producerede fumonisin B_2 og B_4 og på grund af den industrielle brug af denne slægt, bør det undersøges om disse mykotoksiner produceres under industrielle betingelser.

List of original papers

Paper 1: **J.M. Mogensen**, K.F. Nielsen, R.A. Samson, J.C. Frisvad and U. Thrane. Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* spp. BMC Microbiology. 2009. 9:281

Paper 2: **J.M. Mogensen,** J.C. Frisvad, U. Thrane, K.F. Nielsen. Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. Journal of Agricultural and Food Chemistry. 2010, 58:954-958

Paper 3: **J.M. Mogensen**, T.O. Larsen, K.F. Nielsen. Widespread occurrence of the mycotoxin Fumonisin B_2 in wine. Journal of Agricultural and Food Chemistry. 2010, 58:4583-4587

Paper 4: P. B. Knudsen, **J. M. Mogensen**, T. O. Larsen and K. F. Nielsen. Occurrence of fumonisins B_2 and B_4 in retail raisins. Journal of Agricultural and Food Chemistry. 2011, 59:772-776

Paper 5: **J.M. Mogensen**. J. Varga, U. Thrane and J.C. Frisvad. *Aspergillus acidus* from Puerh tea and black tea does not produce ochratoxin A and fumonisin B_2. International Journal of Food Microbiology. 2009. 132:141-144.

Paper 6: **J. M. Mogensen**, E. O. Søndergaard and K. F. Nielsen. Studies on fumonisins in peanuts and peanut butter. Journal of Agricultural and Food Chemistry. Submitted.

Paper 7: **J. M. Mogensen**, S. M. Sørensen, M. Sulyok, L. van der Westhuizen, G. Shephard, J.C. Frisvad, U. Thrane, R. Krska, K. F. Nielsen. Single kernel analysis of fumonisins and other fungal metabolites in maize from South African subsistence farmers. Food Additives & Contaminants. 2011. 28:1724-1734.

Paper 8: **J. M. Mogensen**, K. A. Møller, P. von Freiesleben, R. Labuda, E. Varga, M. Sulyok, A. Kubátová, U. Thrane, B. Andersen, K. F. Nielsen. Production of Fumonisins B_2 and B_4 in *Tolypocladium* species. Journal of Industrial Microbiology and Biotechnology. 2011, 38:1329-1335.

Paper 9: K.F. Nielsen, **J.M. Mogensen**, M. Johansen, T.O. Larsen and J.C. Frisvad. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Analytical & Bioanalytical Chemistry. 2009. 395:1225-1242.

Other papers (non peer review) in Danish and conference proceedings

J.M. Mogensen, J. Mogensen, J.C. Frisvad, U. Thrane, T.O. Larsen and K.F. Nielsen. Giftige fumonisiner fra *Aspergillus niger*. Dansk Kemi. 2009, 90(1):25-27

J. M. Mogensen, P. B. Knudsen, T. O. Larsen, J. C. Frisvad, U. Thrane and K. F. Nielsen. Fumonisin from *Aspergillus niger* in grapes and derived products. Poster at Mycored Africa 2011 (4^{th}-6^{th} April), Cape Town, South Africa

J. M. Mogensen, S. M. Sørensen, M. Sulyok, G. Shephard, L. van der Westhuizen, J. C. Frisvad, U. Thrane, K. F. Nielsen. Fumonisins and other compounds in South African maize–A single kernel approach. Poster at Mycored Africa 2011 (4^{th}-6^{th} April), Cape Town, South Africa

J. M. Mogensen, Peter B. Knudsen, Jens C. Frisvad, Thomas O. Larsen and Kristian F. Nielsen. Fumonisins from *Aspergillus niger* in grapes and derived products. Poster at 32^{nd} Mycotoxin Workshop 2010 (14^{th}-16^{th} june), Lyngby, Denmark.

J.M. Mogensen, J.C. Frisvad, T.O. Larsen and K.F. Nielsen. Fumonisins and other compounds from *Aspergillus niger*. How can an organism be both an industrial workhorse and a food safety problem? Presentation at the 31^{st} Mycotoxin Workshop 2009 (15^{th}-17^{th} june), Münster, Germany

J. M. Mogensen, U. Thrane, J. Varga, T. O. Larsen, J. C. Frisvad and K. F. Nielsen. Fumonisins from *Aspergillus niger* – A new health risk? Poster at Fungi and Health Symposium, Amsterdam, the Netherlands 2008

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1 Introduction

1.1 Fungi & Mycotoxins

Fungal contamination of crops has been a known problem since ancient times. In the 700-800 BC Romans made sacrifices to the god Robigus who had to be appeased in order to protect the grain and trees[1]. The presence of fungi in the field is also mentioned several times in biblical literature, e.g.: "Many times I struck your gardens and vineyards, destroying them with **blight** and **mildew**. Locusts devoured your fig and olive trees, yet you have not returned to me, declares the LORD" (Amos 4:9).

Mycotoxins are secondary metabolites produced by filamentous fungi. Mycotoxins are a diverse range of natural products originating from different biosynthetic pathways. The most common definition of mycotoxins is: "Mycotoxins are natural products produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route."[2,3]. Mycotoxins are distributed worldwide in food and feed products, and are formed under certain environmental conditions e.g. proper moisture, suitable temperature, physical damage to the commodity, and presence of the fungal spores[4-7]. Mycotoxins are produced pre-harvest in the field as well as during postharvest transportation and/or storage [4,5,7,8], causing severe economic losses due to e.g. poor crop quality, yield loss, impaired animal health, and higher mortality rates[9]. As a consequence export of several agricultural commodities, especially crops originating from developmental countries is affected. Good agricultural practices are therefore essential to avoid mycotoxin contamination, because poor harvesting practices, improper drying, handling, packaging, storage, and transport conditions increase fungal growth and mycotoxin production[5].

Diseases caused by mycotoxins, mycotoxicoses, have been known since ancient times and several interpretations have linked historical events to the ingestion of mycotoxins[10-13]. For instance the involvement of mycotoxins in the plague of Athens in 430 BC[14], episodes occurring in the Middle Ages, referred to as "St. Anthony's Fire"[13] and the mycotoxin T-2 toxin as a suspected causative agent for numerous alimentary toxic aleukia related fatalities in Russia during World War II[15]. The fundamental moment for modern mycotoxicology was however, the outbreak of Turkey-X disease in the United Kingdom in 1960, which caused the death of thousands of turkeys and ducklings, and was later attributed to be caused by aflatoxins[16-19]. This event is seen as the cause for the massive

expansion of research efforts in the areas of mycotoxicology, such as mode of action, implication for human health, and chemical determination of new toxins[8]. Based on this huge effort within fungal pathogens of major crops, their natural occurrence and implications for human health, researchers has focused mainly on aflatoxins, fumonisins, trichothecenes, zearalenone, ergot alkaloids, ochratoxin A, and patulin[5,8], figure 1. Although hundreds of fungal toxins are known, a more limited number are generally considered to play an important role in food safety. Mycotoxins of concern are mainly produced by the three genera *Aspergillus*, *Fusarium* and *Penicillium*[20].

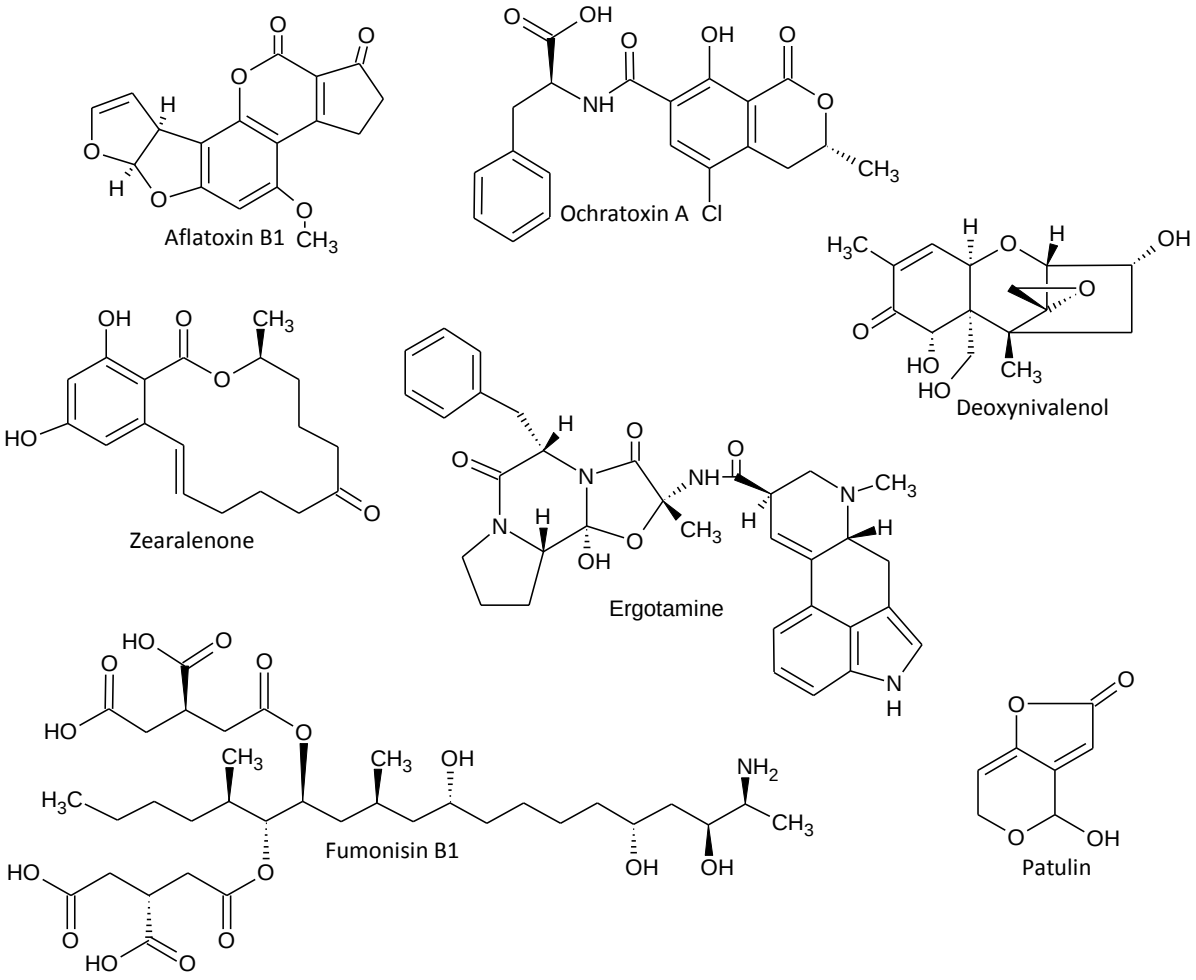


Figure 1: Common regulated toxins.

Within the group of mycotoxins, aflatoxins, ochratoxins, and fumonisins are considered the most important. All three types of mycotoxins are highly regulated, due to their toxicity and prevalence[21]. Aflatoxins are acutely toxic, immunosuppressive, mutagenic, teratogenic and

carcinogenic compounds[4,22]. Aflatoxins are produced by Aspergilli[16,19], and are often associated with maize and peanuts[23,24]. According to USFDA the advisory limit for aflatoxins is 50 µg/kg in all food types (except milk) and EU has regulatory limits of 0.05-15 µg/kg depending on the food type[21,25]. Ochratoxin A has been shown to be nephrotoxic, immunosuppressive, carcinogenic and teratogenic[4,22]. Ochratoxin A are produced by Penicillia and Aspergilli[26,27] and frequently found in cereals, grapes (derived products) and coffee beans[5,23]. The regulatory limits for ochratoxin A are 2-10 µg/kg according to EU[21,28] ; however USFDA has not set any advisory limits for ochratoxin A[29,30]. Fumonisins are cardiotoxic, hepatotoxic, nephrotoxic and associated with several human and animal diseases[31] (elaborated in chapter 1.2.1). Fumonisins are mainly produced by Fusaria (primarily section *Liseola*)[32,33], but *Aspergillus niger*[34-36] is also reported as a producer. Fumonisins are primarily a concern in maize and the EU has set maximum levels of 0.2-2 mg/kg[37] for fumonisins where the USFDA has recommended a maximum levels of 2-4 mg/kg[38]. Regulated and examples of non regulated mycotoxins, their associated commodities along with regulatory levels are given in table 1

Table 1: Examples of mycotoxins; their source, commodities and regulatory limit in food[5,8,13,20,21,23,39,40]

Mycotoxin	Primary source	Important commodities	Regulatory limits in food (µg/kg) USFDA	EU
Aflatoxin	*A. flavus, A. parasiticus*	Maize, peanuts	20	0.01-15
Alternariol	*Alternaria tenuissima*	Cereals	-	-
Citrinin	*P. citrinum, P. expansum*	Grain	-	-
Ergot alkaloids	*Claviceps purpurea*	Grain, primarily rye	-	1000000 (feed)
Fumonisins	*F. verticillioides, F. proliferatum*	Maize	2000-4000	200-4000
Gliotoxin	*A. fumigatus*	Virulence factor in mammalian mycoses	-	-
Ochratoxin	*A. carbonarius, A. ochraceus, P. verrucosum*	Cereals, grapes, coffee beans	-	Food: 0.5-80
Patulin	*P. expansum*	Apples, apple juice	50	10-50
Sterigmatocystin	*A. nidulans, A. versicolor*	Moldy grain, green coffee beans and cheese	-	-
Deoxynivalenol	*F. equiseti, F. graminearum*	Cereals	1000	200-1750
Zearalenone	*F. graminearum, F. culmorum*	Cereals	-	20-400

1.2 Fumonisins

The fumonisins were first isolated from *Fusarium verticillioides* (=*Fusarium moniliforme*) by Bezuidenhout *et al.* in 1988[33]. Analogues from *Fusarium* have been identified since and are classified in four structural series of fumonisin designated A, B, C, P (figure 2)[33,41,42]. The four

groups have the same scaffold but differ in selected structural moieties. The A-groups differ from the B-group by the amine which is acetylated, the C-group consist of a C19 backbone instead of C20, the P-series have a 3-hydroxypyridinium group instead of the amino group at position C2 [33,41,42]. The B-series is the most abundant naturally occurring fumonisin with FB_1 as the predominant[32,43].

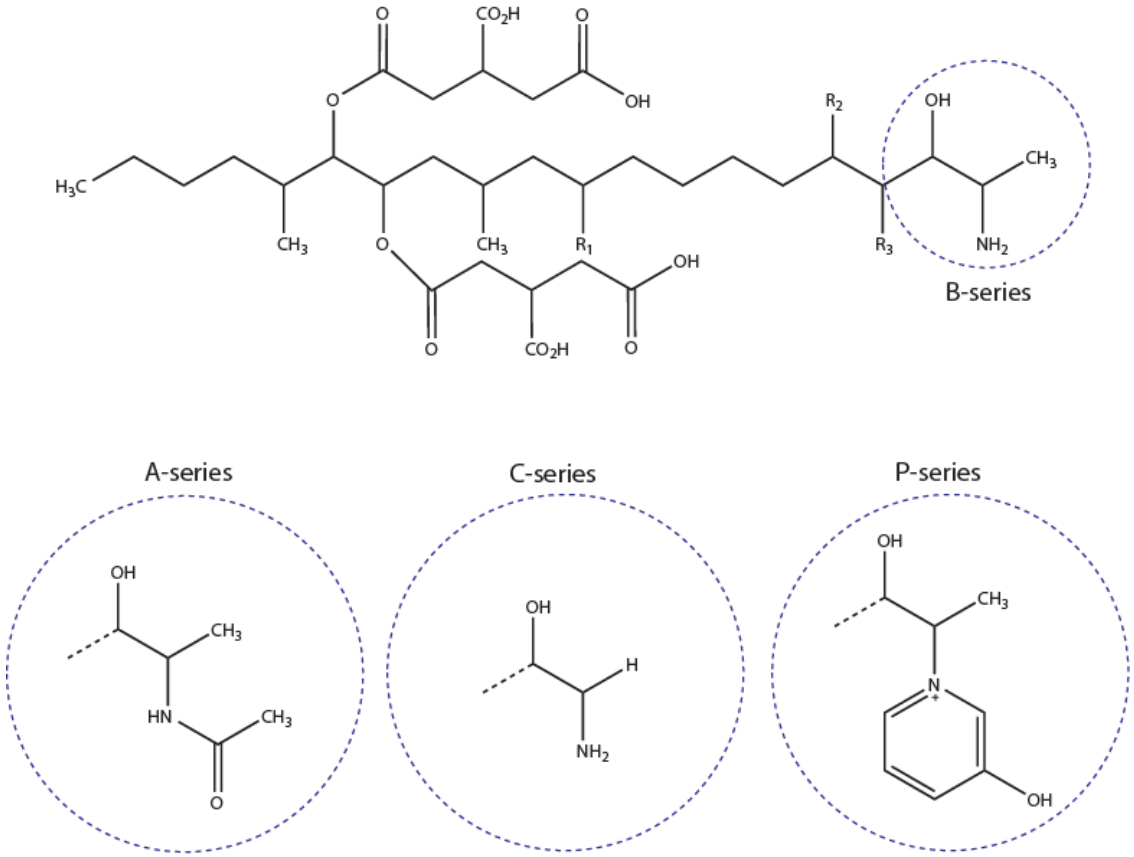


Figure 2: Overall structure of fumonisin A, B, C, P-series.

The event that led to the discovery of the fumonisins were a field outbreak of equine leukoencephalomalacia (ELEM) in South Africa in 1970[44]. During the investigation of this event, *F. verticillioides* was isolated from a batch of moldy maize believed to be the causative agent of the ELEM outbreak[45]. Focusing the research on this target organism (*F. verticillioides*), showed that a number of these caused ELEM in horses, pulmonary edema in pigs and was highly hepatoxic and cardiotoxic in rats [46,47]. In 1988, a breakthrough came with the isolation FA_1, FA_2, FB_1, and FB_2[33]. In the following years, several *Fusarium* species were identified as fumonisins producers[32,43,48] as

well as identification and structural elucidation of numerous fumonisins analogs[42,49,50]. The fumonisin producing Fusaria are primarily found within section *Liseola,* mainly *F. proliferatum* and *F. verticillioides*[32], however species of section *Discolor, Elegans, Gibbosum,* and *Martiella* are also reported as fumonisin producers[34,48,51]. A single report has furthermore claimed that *Alternaria arborescens* (reported as *A. alternata*) produced FB_2, but this strain has since lost this ability[52-54]. In 2006, a surprising discovery was made, when full genome sequencing of *A. niger* showed that the genome included a putative *Fum* gene cluster[55,56]. This gene cluster could be silenced and hereby not expressed, but in 2007 it was shown that *A. niger* is able to produce FB_2, and this was followed by the discovery of production of FB_4 and the new FB_6[34-36]. In total fumonisin production has been reported in 26 *Fusarium*, one *Alternaria* and one *Aspergillus* species, table 2.

Table 2: Species reported as fumonisin producers and their production of fumonisins

Species	Fumonisins	References
Fusarium acutatum	B_1, B_2	[57]
F. andiyazi	B_1, B_2	[58-60]
F. anthophilum	B_1, B_2	[51,61]
F. begoniae	B_1	[57]
F. brevicatenilatum	B_1	[57]
F. dlamini	B_1	[51]
F. equiseti	B_1	[48]
F. fujikuroi	B_1, B_2, B_3	[60,62,63]
F. globosum	B_1, B_2, B_3	[62,64]
F. lactis	B_1	[65]
F. napiforme	B_1	[51]
F. nygamai	A_1, A_2, A_3, B_1, B_2, B_3, B_4, B_5, C_1	[43,51,58,66,67]
F. oxysporum	B_1, B_2, B_3, C_1, C_4	[41,48,68]
F. phyllophilum	B_1	[57]
F. polyphialidicum	B_1	[69]
F. proliferatum	A_1, A_2, A_3, B_1, B_2, B_3, C_1, C_4, P_1, P_2, P_3	[51,66-68]
F. pseudocircinatum	B_1, B_2	[57]
F. pseudonygamai	B_1, B_2	[58]
F. ramigenum	B_1, B_2	[70]
F. redolens	B_1, B_2, B_3	[71]
F. sacchari	B_1	[59,72]
F. sambucinum	B_1	[73]
F. solani	B_1	[73]
F. subglutinans	B_1, B_2, B_3	[63,72]
F. thapsinum	B_1, B_2, B_3	[58,59,74]
F. verticillioides	A_1, A_2, A_3, B_1, B_2, B_3, B_4, B_5, C_1, C_3, C_4, P_1, P_2, P_3	[33,58,66,68]
*Alternaria arborescens**	B_1	[52]
Aspergillus niger (and *A. awamori*)	B_2, B_4, B_6	[34,35]

*reported as *Alternaria alternata* (Fr.) Kiessl. f. sp. *lycopersici*. This strain has since lost its ability to produce FB_1

1.2.1 Toxicity of fumonisins

Numerous toxicity studies of fumonisins have been performed, using various assays to determine the different types of toxicity. This is important due to the link to both human and animal toxicoses from consumption of contaminated maize-based food and feeds[75]. As the most abundant fumonisin, FB_1 is the most thoroughly investigated concerning the toxicity of fumonisins. The causality between fumonisins and animals has been proved in several cases[31,76]. Fumonisin and *F. verticillioides* contaminated feeds have been recognized as the cause for the neurotoxic disease ELEM in horses[77,78], and are furthermore cardiotoxic and cause pulmonary edema in pigs[79]. Cattle and poultry appear to be less sensitive than horses, pigs, rabbits and laboratory rodents[31], resulting in a less strict guidance levels in feed specified for these[38]. It has been shown in laboratory animals that liver and kidney are the major target organs; however, species-, strain- and sex-dependent differences in dose response occur[31,76,80].

The effect of fumonisins on human health is uncertain[76]. Epidemiologic studies around the world have revealed that the incidence of human esophageal cancer and the occurrence of *F. verticillioides* appear to correlate[31]. Furthermore, fumonisins are suspected risk factors for liver cancer[81,82], neural tube defects[83-86], and cardiovascular problems[87] in populations with a high intake of food made with contaminated maize.

Concerning the genotoxic potential of fumonisins, several independent studies have been performed; however, with conflicting results, since reports of positive and negative results in various assays (both *in vivo* and *in vitro*) have been published[31].

Fumonisins are structurally similar to the sphingolipids precursors[88]. These similarities cause a disruption/inhibition of the sphingolipid biosynthesis, specifically the ceramide synthase[88]. It is particular fumonisins with an amino group at C2, which competitively inhibit ceramide synthase, and hereby disrupt the *de novo* biosynthesis of ceramide and sphingolipid metabolism [89,90]. Inhibition of biosynthesis of sphingolipids is seen at different levels, and is reflected in changes of the ratio sphinganine/sphingosine (Sa/So)[91]. Although the specific effects of sphingolipids on apoptosis are complex and not fully understood, there is no doubt that in various in vivo and in vitro cellular systems, administration of FB_1 produces alterations in sphingolipids with consequent alterations of apoptosis and cell proliferation[92]. The results suggesting that the amino group appears to be the reason for the ceramide synthase inhibition and toxicity of fumonisins, appear

to be true since N- substituted fumonisins showed reduced toxicity both *in vivo* and *in vitro*[93-95] and less ceramide synthase inhibiting ability[96].

Since the molecular structures of FB_2 and FB_3 are almost identical to FB_1, it has been claimed that the degree of toxicity may be at the same level as that of FB_1[94,97,98]. From a *Fusarium* point of view, the importance of their toxicity is marginal, since the levels of these compounds are usually 4-10 times lower than FB_1. However with the production of FB_2, FB_4 and FB_6 by *A. niger* further research about these compounds is necessary.

1.2.2 Analysis of fumonisins in food & feed

The widespread prevalence and toxicity of fumonisins resulted in the development of a number of analytical methods for detection of fumonisin levels in food and feeds, table 3. In the first report of the isolation of fumonisins, Gelderblom *et al.* used normal phase chromatography as well as HPLC[99]. Since then, the need for quantitative analytical methods for detection of fumonisins has been obvious. Initially the primary focus was on gas chromatography[100-103]. GC-MS analysis is based on hydrolysis of the esterified side chain and trimethylsilyl or trifluoroacetate derivatization, and is sensitive and selective, but at the time involved expensive equipment[104].

Table 3: Detection methods used in fumonisin analysis and examples of their advantages/disadvantages

Detection method	Chemical modification	Advantages	Disadvantages
HPLC-FLD	Derivatization of the amine	sensitive, widespread , equal response factor, low initial cost	Derivatives decompose quickly leading to underestimation, derevatization of every amine group, high running costs, clean-up procedures
GC-MS (rare)	Hydrolysis and esterification	Sensitive, selective	Complicated pre-treatment, multiple handling steps
TLC	Ninhydrin, *p*-anisaldehyde or flourescamine spray	Cheap, simple, rapid	Lack of automation, sensitivity, Verification by GC or LC
ELISA	None	Easy, rapid, inexpensive, Sensitive, specific	Overestimates fumonisin level, less sensitive than LC, cross reactivity, Verification by GC or LC
LC-MS	None	No chemical modification, less sample prep than other, crude extract can be analyzed, high sensitivity	Expensive equipment, ion-suppression

However focus shifted towards HPLC methods, due to the multiple handling steps (hydrolysis, clean-up, derivatization) prior to GC-MS analysis[105]. Since HPLC methods were easier to apply and required less sophisticated equipment, they became the method of choice for detection and quantification, primarily HPLC coupled to a fluorescence detector (FLD)[105,106]. The fumonisins are highly polar molecules (logD -1.5 at pH 3.2 and -4.21 at pH 7) which are soluble in water and polar solvents, and are suited for separation by reversed phase HPLC[105] (acidic). Since fumonisins lack UV absorption, and therefore undetectable with UV or FLD, chemical modification like derivatization of the amine with o-phthaldialdehyde (OPA) is necessary to make it detectable[107]. However the disadvantages of OPA derivatives of fumonisins are, that they decompose very rapidly, thus leading to underestimation of the actual level of contamination[108]. Additionally, OPA may react with other amino group containing compounds, resulting in some interference[108]. Chromatographic detection has mostly been achieved with a UV and fluorescence detector, advances within liquid chromatography–mass spectrometry (LC–MS) technology have however, shifted the emphasis towards this analytical approach[105,108,109]. The LC-MS provides quantitative analyses as well as confirmation of the identity of the fumonisins without any need of derivatization. Moreover, the commercial availability of the isotopically labeled FB_1 allowed the development of LC/MS methods based on the isotopic dilution approach, which are usually characterized by high accuracy[108,110]. Furthermore virtually all mycotoxin analytes are compatible with LC-MS because of the conditions applied during separation and detection[109,110]. Electro Spray Ionisation (ESI)-MS is an ideal way to ionize, detect, and measure fumonisins, which are ionic compounds and therefore produce abundant signals in both positive and negative ion modes, however positive ion mode is most commonly used [108,110,111]. Compared to the other detection techniques, the major advantage of the LC/ESI/MS detection of fumonisins is beside that no derivatization or special sample preparation is required, no fragmentation is produced during the ionization[108,110,111]. For these reasons, it is possible to directly analyze culture materials and food products by simply extracting, filtering, and injecting the sample onto the LC column. Additionally, a recent comparison of fumonisin detection using HPLC-FLD, LC-MS and LC-MS/MS was reported, proving that LC-MS/MS had the highest sensitivity followed by HPLC-FLD and LC-MS[112].

Immuno based techniques such as ELISA have also been used for detection of fumonisins in food and feed[8,105,107,109,110]. The advantages of this approach is that it is easy, rapid and

inexpensive[107], however ELISA methods generally overestimate the fumonisin content in samples and positive samples needs verification by other means[107,108].

Often the crude extracts must be purified in order to remove matrix components and to concentrate the fumonisins prior to analytical chromatographic analysis. Examples of sample clean-up with different approaches, involved the usage of reversed phase, strong anion-exchange (SAX) or immunoaffinity columns (IAC) [113,114]. IAC provides the most selective purification, and the user obtain cleaner extracts with a minimum of interfering matrix components and leading to excellent signal-to-noise ratios compared to the use of the less selective SPE sorbent materials[110]. However, IAC have a lower binding capacity (1-2 µg FB's) and therefore IAC is not recommendable for purification and determination of high amounts of FB[105,107]. SAX columns have showed a superior purification compared to reversed C18 phase columns[115]; however, SAX require a pH of the sample extract above 5.8 for adequate retention[116].

1.2.3 Fumonisins in Food & Feed – Recent findings

Fumonisins are found frequently in maize, and almost all reported findings during the last 30 years are reported in maize [3,31,117,118]. The reported concentrations vary a great deal [3,117], with levels up to 300 mg/kg observed in Italy[31,119]. Although fumonisins in maize and derived products are the primary problem, reports of fumonisins in other commodities have been published, for instance rice[120], sorghum[121], black tea[122] and beer[123]. Table 4 illustrates examples of recent findings of fumonisins in food commodities other than maize and maize products.

Table 4: Examples of recent findings (2005-2011) of fumonisin B_1 associated with *Fusarium* in non-maize products

Food type	Country	Frequency	Levels (µg/kg)	References
Asparagus	China	80% (n=80)	24-670	[124]
Beans	Cameroon	20% (n=15)	28-1351	[125]
Beer	European	97% (n=33)	0.1-30.3	[126]
Cereals	Malaysia	19% (n=100)	41.3-209.3	[127]
Dried figs	Turkey	75% (n=15)	0-3649	[128]
Milk	Italy	80%	0.23-0.46	[129]
Oats	Argentina	9% (n=23)	105-108	[130]
Peanuts	Cameroon	19% (n=16)	25-1498	[125]
Retail food	Japan	23% (n=810)	0-1380	[131]
Rice	Canada	15%	4.5 (Mean)	[132]
Sorghum opaque beers	Cameroon	88% (n=120)	0.0-230	[133]
Spices	Tunisia	15% (n=13)	70-130	[134]
Tea	China/Belgium	1% (n=91)	76	[135]

With the recent finding of a fumonisin production in *A. niger*[34-36], new food commodities are at risk. Until now there are very few reports of fumonisins associated with *A. niger* in food and feed, these are shown in table 5. A general trend of these fumonisin levels are that levels associated with *A. niger* are markedly lower than levels in commodities associated with *Fusarium.*

Table 5 Findings of fumonisin B_2 associated with *Aspergillus niger* in food.

Food type	Country	Frequency	Levels (µg/kg)	References
Raisin	6 different countries	54% (n=13)	157-11516	[136]
Grape must	Italy	15% (n=12)	10-400	[137]
Wine	Italy	18% (n=51)	0.4-2.4	[114]
Coffee beans	Thailand	58% (n=12)	1.3-9.7	[36]

1.3 Black Aspergilli

1.3.1 Occurrence & taxonomy

The genus *Aspergillus* consists of more than 250 species[138]. Within the genus are the black Aspergilli (*Aspergillus* subgenus *Circumdati* section *Nigri*), which are an important group in food mycology and biotechnology. Members of section *Nigri* are ubiquitous and have been isolated from a wide variety of substrates found around the world[139-143]. The section *Nigri* consists of 25 species including both uniserate and biserate species (table 6) of which *A. acidus*, *A. brasiliensis*, *A. carbonarius*, *A. ibericus*, *A. niger,* and *A. tubingensis* are found commonly[144-146]. The rest of section *Nigri* are rare and each species is suggested to represent a frequency below 1%[146]. Within section *Nigri, A. niger* is the most common contaminants of food[146], especially fruits and certain vegetables[143], and is frequently determined in grapes, green coffee beans, onions, mango, maize and other cereals, peanuts, dried fruits, and many other products[36,143,147].

Table 6: Overview of *Aspergillus* section *Nigri*. (Bold: Most common)[144,145]

Clade	Species
A. niger clade	***A. acidus***, *A. awamori*, ***A. brasiliensis***, *A. costaricaensis, A. eucalypticola, A. neoniger,* ***A. niger***, *A. piperis,* ***A. tubingensis***, *A. vadensis*
A. carbonarius clade	***A. carbonarius***, ***A. ibericus***, *A. sclerotiicarbonarius, A. sclerotioniger*
A. heteromorphus clade	*A. ellipticus, A. heteromorphus*
A. homomorphus clade	*A. homomorphus, A. saccharolyticus*
A. aculeatus clade	*A. aculeatinus, A. aculeatus, A. fijiensis, A. indologenus, A. japonicus, A. uvarum, A. violaceofuscus*

The taxonomy of section *Nigri* is one of the most confusing and complex in the fungal world due to the subtle differences between the species, which is underlined by several proposed taxonomic

schemes[140,141,144,148,149]. As a result several reported strains have incorrectly been named *A. niger*, because the organism is seen as a black Aspergilli and therefore reported as *A. niger*[146]. Using morphological structures can be a helpful tool when identifying black Aspergilli, especially a species, such as *A. carbonarius* (size of conidia) and the uniserate species (e.g. *A. japonicus, A. aculeatus*) are easily recognized using identification manuals[144,145]. In contrast, species related to *A. niger* are very difficult to distinguish[144]. A polyphasic identification approach, in which morphology, metabolite profiling, and/or several molecular methods (sequence data of the β-tubulin, calmodulin and ITS regions) are used, is thus preferred[144,145,150]. An example of the problematic identification of species within section *Nigri* was given in a recent comparison of the β-tubulin, calmodulin and ITS sequence data from all described members of the section *Nigri*. This comparison showed that *A. foetidus* and *A. lacticoffeatus* are synonyms of *A. niger* and *A. coreanus* is synonym of *A. acidus*[145].

1.3.2 The industrial workhorses

The black Aspergilli are able to produce a wide range of metabolites, as listed in section 1.3.3. Also, the black Aspergilli have a number of characteristics which make them ideal organisms for industrial applications as high levels of protein secretion and ability to assimilate various organic substrates [151]. Therefore, many species of the black Aspergilli are used in the fermentation industry to produce hydrolytic enzymes, such as amylases or lipases, and organic acids such as citric acid and gluconic acid[152,153]. The versatile metabolism of *A. niger* has made it one of the most important production organisms for industrial fermentation[56,152], and after thorough research *A. niger* used with known industrial processes, has been placed on the Generally Recognized as Safe (GRAS) list of the USFDA[154].

The first industrial use of *Aspergillus niger* was in 1919, when its ability to produce citric acid was exploited. *A. niger* is the preferred choice of microorganism for the citric acid bio-production process, due to a high ease of handling/harvesting, extraordinary ability to ferment a variety of raw materials (e.g. agricultural waste residues), and high product yields[155]. Strain improvement and optimization of the production media have resulted in a process of citric acid accumulation by which cells convert up to 80% of the substrate, sucrose, to the final product[156].

A. niger and *A. awamori* are well established expression platforms for the production of secreted proteins and enzymes[157]. In the natural environment these fungi are involved in the degradation of plant cell material by producing highly specialized enzymes such as pectinases, cellulases, and xylanases[151]. *A. niger* is additionally considered the ancestor of many currently used enzyme production strains[56], and has been used as a rich source of a variety of enzymes well established in fruit processing, baking, and the starch and food industries since the 1960s. The first to be utilized were pectinase, protease, and amyloglucosidase[152].

1.3.3 Secondary metabolism

The secondary metabolism of the black Aspergilli is wide and diverse, covering alkaloids, polyketide and terpenes. More than 145 metabolites have been isolated and structure elucidated from the biserate black Aspergilli[158] a thorough review of this is given in paper 9. Examples of secondary metabolites produced by members of section *Nigri* are given in figure 3

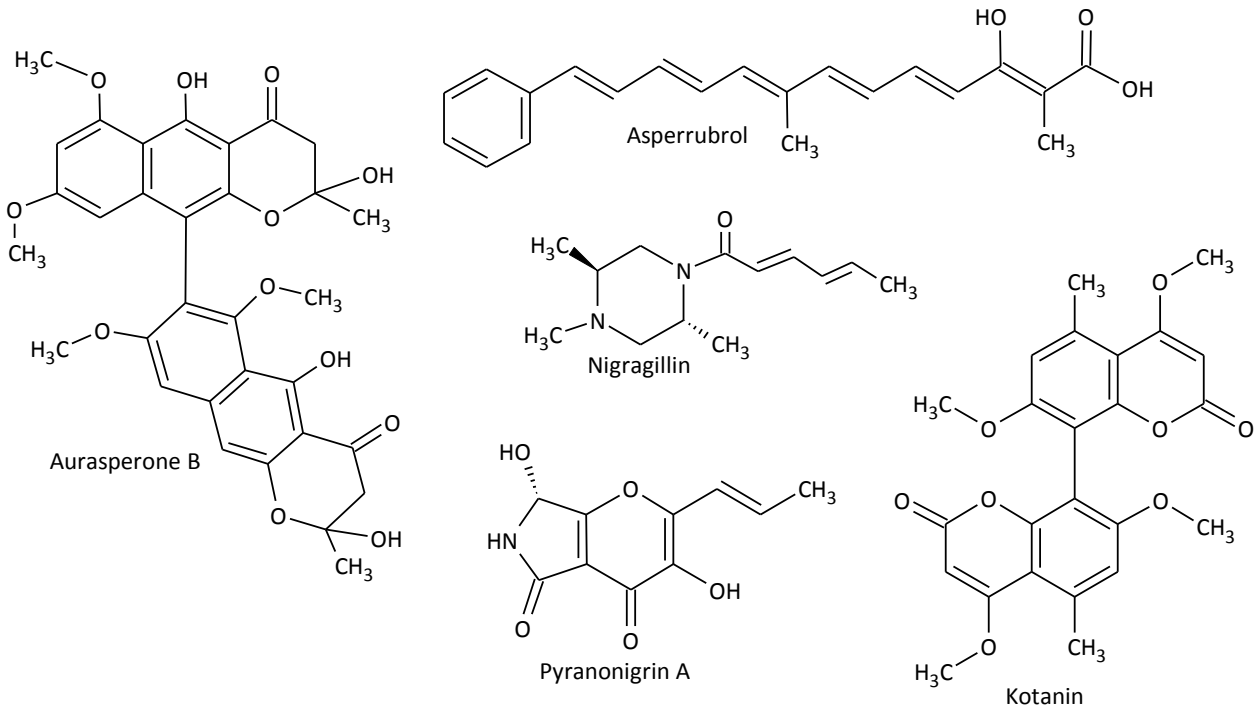


Figure 3: Examples of secondary metabolites produced within Section *Nigri*.

Several guidelines to distinguish between the species using the secondary metabolite profile can be used, since there are several differences among the species. Table 7 gives an overview of the most common secondary metabolites produced by black Aspergilli.

Table 7: Production of secondary metabolites in section *Nigri*[139,144-146,159]

Species	Extrolite
A. acidus	antafumicins, asperazine, funalenone, naphtha-γ-pyrones, pyranonigrin A, nigragillin
A. aculeatinus	Aculeasins, neoxaline, secalonic acid D, secalonic acid F
A. aculeatus	secalonic acid D, secalonic acid F
*A. awamori**	Same as *A. niger*
A. brasiliensis	naphtho-γ-pyrones, pyrophen, tensidol A & B
A. carbonarius	ochratoxins (A, B, α, β), naphtho-γ-pyrones, pyranonigrin A
A. costaricaensis	aflavinines, funalenone, naphtho-γ-pyrones
A. ellipticus	austdiol, candidusins, terpenyllin, cf. xanthoascin
A. eucalypticola	Pyranonigrin A, funalenone, naphtho-γ-pyrones,
A. fijiensis	asperparalins, secalonic acid D, F
A. heteromorphus	lots of highly unique extrolites including indol-alkaloids, none of them structure elucidated
A. homomorphus	dehydrocarolic acid, secalonic acid D, secalonic acid F
A. ibericus	naphtho-γ-pyrones, pyranonigrin A
A. indologenus	okaramins A, B, H
A. japonicus	cycloclavine, festuclavine
A. neoniger	funalenone, naphtho-γ-pyrones, pyranonigrin A
A. niger	Fumonisin B_2, B_4, B_6, funalenone, kotanins, malformins, naphtho-γ-pyrones, nigragillin, ochratoxin A, pyranonigrin A, tensidol A & B
A. piperis	aflavinins, naphtho-γ-pyrones, pyranonigrin A
A. saccharolyticus	None known
A. sclerotiicarbonarius	naphtho-γ-pyrones, pyranonigrin A, three unique indol-alkaloids at retention indices 1475, 1676 and 1838.
A. sclerotioniger	corymbiferan lactones, funalenone, naphtho-γ-pyrones, ochratoxins (A, B, α, β), pyranonigrin A
A. tubingensis	asperazine, funalenone, malformins, naphtho-γ-pyrones, nigragillin, pyranonigrin A, tensidol A & B
A. uvarum	asterric acid, dihydrogeodin, erdin, geodin, secalonic acid D and F
A. vadensis	asperazine, naphtho-γ-pyrones, nigragillin, a polar orlandin-like compound
A. violaceofuscus	indol-alkaloids

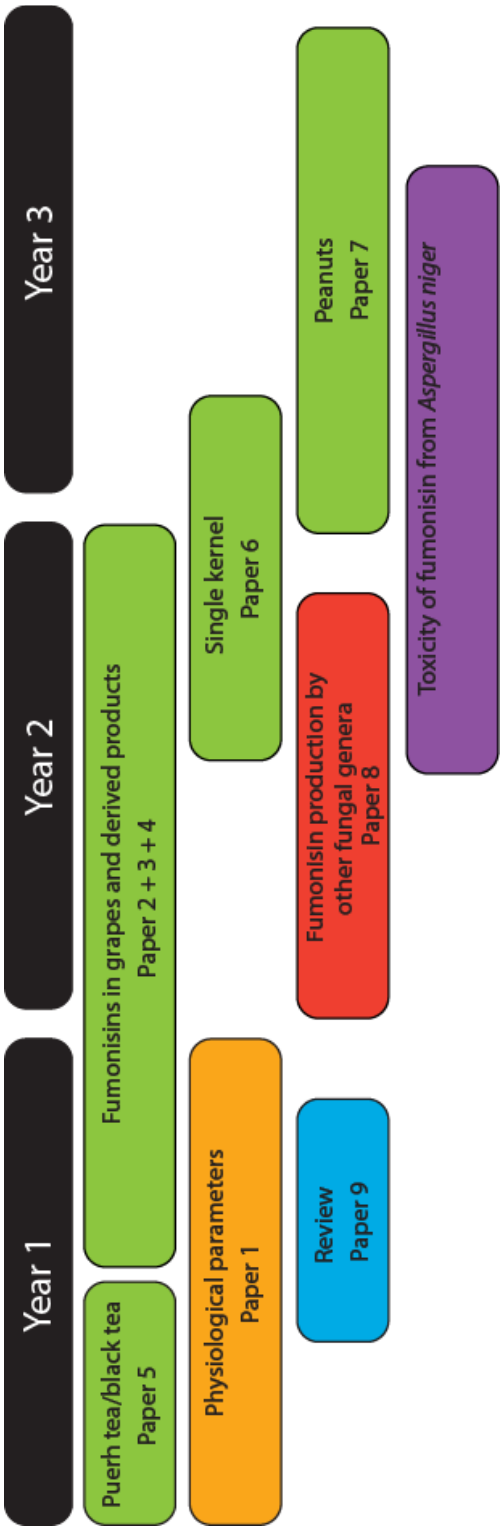
*: only a phylogenetic species

The economical most important secondary metabolite within section *Nigri* is ochratoxin A and its precursors due to its high prevalence in food and feed and toxicity. The main organisms producing this compound are *A. carbonarius* and *A. niger*; however, production of ochratoxin A by *A. japonicus*[160,161], *A. sclerotioniger*[139], and *A. tubingensis*[162] has also been reported, although those of *A. japonicus* and *A. tubingensis* are questioned. *A. carbonarius* is seen as a consistent ochratoxin A producer, contrary to *A. niger* of which roughly 10-15 % produce this mycotoxin at 10-1000 lower amounts[146,163,164]. Another major group of mycotoxins, produced within section

Nigri is the fumonisins; however, so far only *A. niger* is reported as a producer of this compound. Other species tested with a negative result are *A. acidus,A. aculeatinus, A. aculeatus, A. brasiliensis, A. carbonarius, A. costaricaensis, A. ellipticus, A. heteromorphus, A. homomorphus, A. ibericus, japonicus, A. piperis, A. sclerotiicarbonarius, A. sclerotioniger, A. tubingensis, A. uvarum,* and *A. vadensis*[137,146,165]. This means there are still 6 species that have not been screened for a fumonisin production, all newly described[145]. Several reports indicate that 60-80% of *A. niger* strains are able to produce fumonisins[137,165], indicating that the fumonisin production by *A. niger* is more significant than OTA from *A. niger*. Since *A. niger* are able to produce both mycotoxins, a simultaneously production can occur, and approximately 10% are able to produce both mycotoxins[146]

2 Overview of experimental work

This figure is intended as an overview of the main experiments performed during the period. The obtained results will be discussed in the following chapters. The experimental design is found in the denoted papers.



3 Results and Discussion

3.1 Comparative physiological studies of fumonisin production in *Fusarium* and *Aspergillus niger*

When this project was initiated, the knowledge and understanding of the fumonisin production by *A. niger* was sparse. It was only known that *A. niger* produced FB_2 preferably on media with a high sugar/salt content[34]. Therefore, it seemed relevant to investigate how physiological parameters like temperature and water activity affected fumonisin production by *A. niger*.

To compare *A. niger* and *Fusarium*, 25 *Fusarium* strains and five *A. niger* strains were inoculated on three different media, Czapek yeast extract agar + 5% NaCl (CYAS)[166], Rice meal corn steep liquor agar (RC)[167], potato dextrose agar(PDA)[147] and incubated for seven days at 25 °C. The *A. niger* strains consistently produced FB_2 on all three media favoring RC slightly over CYAS and with a poor production on PDA, table 8. These results partly agree with what Frisvad *et al*.[34] stated, CYAS only supported fumonisin production by *A. niger* and RC supports fumonisin production by both genera. Fumonisin production was detected in all five *A. niger* strains on PDA, which is contradictory to the study by Frisvad *et al.* where no fumonisin production was detected, though they only tested one strain and the lack of fumonisin production could be explained by levels were below the detection limit or strain differences, as our results indicate occurs in *Fusarium* species.

Table 8: Fumonisin production by *Aspergillus niger* (FB_2) and *Fusarium* spp. (FB_1, FB_2, FB_3) on CYAS, PDA and RC after 7 days growth at 25°C. The replicates are made in biological duplicates on two separate plates.

Fungi	CYAS µg/ml	RC µg/ml	PDA µg/ml
A. niger (n=5)	2.9-25 (5/5)	5.3-35 (5/5)	0.46-3.1 (5/5)
F. proliferatum (n=6)	n.d. (0/6)	0.028-46 (6/6)	2-33 (4/6)
F. verticillioides (n=6)	n.d.(0/6)	0.028-2.2 (6/6)	4.5-35 (5/6)
F. dlaminii (n=2)	n.d. (0/2)	n.d. (0/2)	n.d. (0/2)
F. napiforme (n=2)	n.d. (0/2)	0.13-0.24 (2/2)	0.081-6.2 (2/2)
F. nygamai (n=8)	n.d. (0/8)	0.01-22(8/8)	0.14-16 (6/8)
F. oxysporum (n=1)	n.d. (0/1)	2.78 (1/1)	37 (1/1)

CYAS: Czapek Yeast extract Agar + 5%NaCl; PDA: Potato Dextrose Agar; RC: Rice meal corn steep liquor agar.
Standard deviation calculated on two measurements
n.d. not detected.

For further analysis, the five *A. niger* and one randomly selected strain from each *Fusarium* species were inoculated on CYAS and PDA respectively and incubated at six different temperatures between 15-42 °C for 7 days. Fumonisin production occurred at temperatures between 20-37 °C

(figure 4), with four strains having a maximum production at 25 °C and the latter at 30 °C. At 42 °C all *A. niger* strains grew, but had no detectable fumonisin production. Thus production of fumonisin seem to follow a similar pattern to that of ochratoxin A[168]. However on a synthetic grape medium and maize kernels ochratoxin production were largest at 15 °C[169,170].

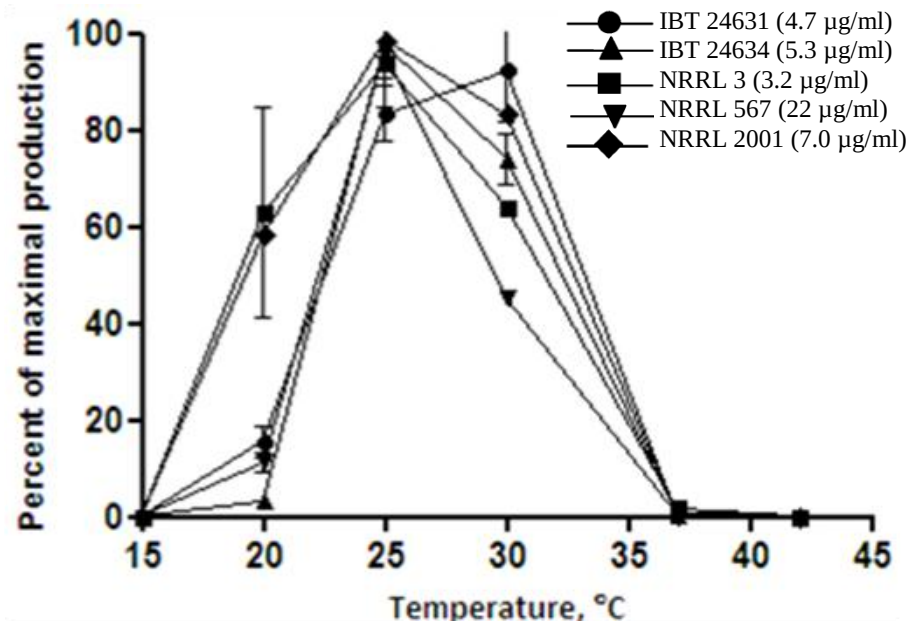


Figure 4: FB_2 production by *A. niger* (A) after 7 days of growth at different temperatures (15-42°C) on CYAS. The concentration of FB_2 was detected in the methanol:water (3:1) extract. The values are means of biological duplicates on two different plates; highest value (µg/ml) is in parenthesis. (PAPER 1)

Fumonisin production at different a_w by two of the five strains (IBT 24634 and NRRL 567) is given in figure 5. All five strains had decreasing fumonisin production, with decreasing water activity, when grown on glycerol. On sucrose both had an increase in their production; however, below 0.99 IBT 24634 had decreasing production, ending below the production at starting conditions. NRRL 567 had more or less constant production below 0.995. On NACl a different picture was seen, NRRL 567 had a slight increase followed by a decreasing production. IBT 24634 had a relatively constant production, with no variations until 0.94 after which it stops. In agreement with our results, Frisvad *et al*. found that the addition of 5 % NaCl (aw = 0.97) or 20 % sucrose (aw = 0.99) increased the production of FB_2 by *A. niger*[34].

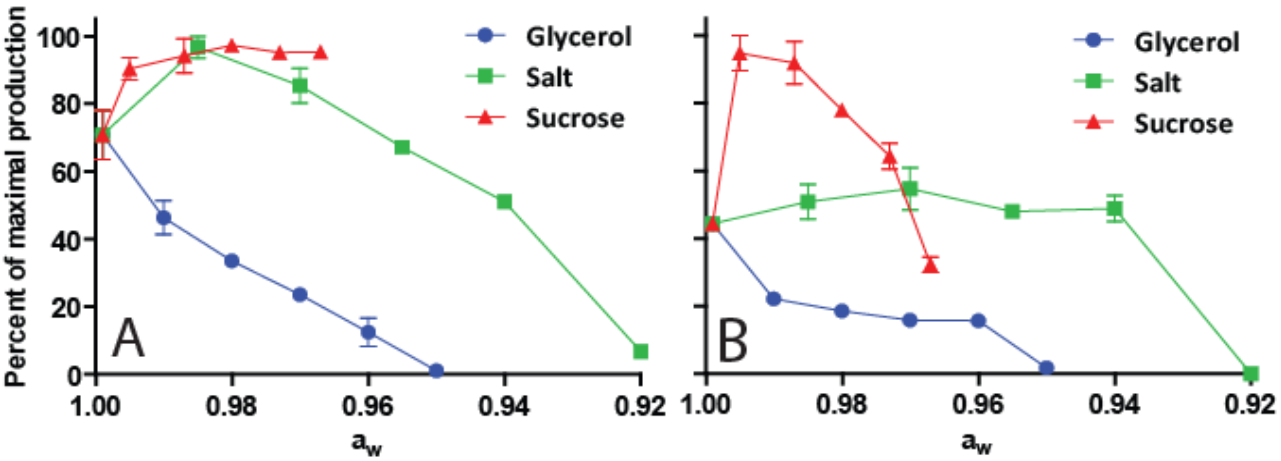


Figure 5: Effect of glycerol, NaCl and sucrose on the FB_2 production of *A. niger*, A: NRRL 567 (max: 22 µg/ml) B: IBT 24634 (max: 5.3 µg/ml). Isolates have been incubated at 25 ˚C for 7 days on CYA. The concentration of FB_2 was detected in methanol:water (3:1) extracts. The values are means of biological duplicates on two different plates. (PAPER 1)

As this is currently the only report of the effect of water activity on the production of fumonisins by *A. niger*, a comparison is made to the production of ochratoxin A by *A. niger*. Previous studies of the optimal water activity of the production of ochratoxin A are inconclusive, stating both 0.95 and the range 0.96-0.99[170,171]. These are different from our results, with four strain preferring 0.99 and the last 0.98. Each strain also responded differently to the various solutes, with highest production on sucrose, followed by NaCl and glycerol.

Earlier studies of the optimal a_W for fumonisin production by *Fusarium,* showed that it favored 0.97-0.98[172,173]. Our results furthermore indicate, that the regulation of the fumonisin production in *A. niger* and *Fusarium* are very different, with the latter preferring high a_w (>0.99) and lower temperatures (20-25 °C), compared to *A. niger* which prefers lower a_w and higher temperature (25-30 °C).

3.2 Fumonisin from *Aspergillus niger* in food

3.2.1 Production of fumonisin in grapes and derived products

Due to the presence of ochratoxin A in grapes and derived products, black Aspergilli have already been identified as the causative organisms in these commodities[174-176]. The fumonisin contamination of grapes and their derived products was obviously relevant to investigate. In order to determine the distribution of fumonisins producing *A. niger* in raisins, black Aspergilli were isolated from 17 different raisin brands. Altogether 86 black Aspergilli were isolated and 66 were

identified as *A. niger*, 4 *A. tubingensis*, and 16 *A. acidus*. These isolates were screened for potential fumonisin production on three different media, Czapek yeast extract agar (CYA)[166], yeast extract sucrose agar (YES)[166] and CYAS[166]. Neither *A. tubingensis* nor the *A. acidus,* produced detectable amounts of fumonisins, as opposed to 77 % of the *A. niger* strains, that were able to produce fumonisins. This frequency was similar to that reported for *A. niger* from green coffee beans[36] and grapes[137], as well as industrial *A. niger* strains[146]. Though higher than those reported from Californian raisins[165] and Portuguese grapes[177]. The strains isolated in the Portuguese grape study were only identified as *A. niger* aggregate, which explains the lower frequency, since this also covers other common black Aspergilli like *A. tubingensis* and *A. acidus*. The abundance of fumonisin producing *A. niger* confirmed that a potential fumonisin contamination was present. Worst case scenarios were performed by inoculation of ten isolates on grapes. All ten isolates were able to produce fumonisins on the grapes, table 9. Surprisingly none of the ten isolates were able to produce ochratoxin A under these circumstances.

Table 9: Production of fumonisins by *Aspergillus niger* in grapes after 7 days growth at 25˚C. (PAPER 2)

Isolate IBT	Fumonisin B_2 µg/kg		Fumonisin B_4 µg/kg[A]		Fumonisin B_6 µg/kg[B]		Ochratoxin A µg/kg[C]
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2	
28747	951	1283	75	189	n.d.	n.d.	n.d.
28753	7703	7979	772	1542	117	129	n.d.
28934	2098	5376	158	612	5	77	n.d.
28937	1045	1941	253	265	n.d.	n.d.	n.d.
28948	3380	4772	349	509	63	65	n.d.
28964	91	251	5	23	n.d.	n.d.	n.d.
28965	1074	2414	148	344	28	n.d.	n.d.
28966	1261	5123	220	634	60	n.d.	n.d.
28994	432	2422	36	158	n.d.	n.d.	n.d.
29019	4303	11189	143	405	89	135	n.d.

[A]: Assuming same response factor as fumonisin B_2. [B]: Assuming same response factor as fumonisin B_1. [C]:LOD: 3 µg/kg. nd: not detected, LOD: 5 µg/kg

All strains produced FB_2 and FB_4, whereas only six strains produced FB_6, the average ratio of these is ~89% FB_2, ~10 % FB_4 and ~1 % FB_6. The FB_2 levels in grapes were 171-7841 µg/kg, FB_4 were 14-1157 µg/kg, and FB_6 levels were 5-135 µg/kg. These fumonisin levels are comparable to a similar study by Logrieco *et al.*[137].

To investigate how/if the fumonisin production could occur in raisins, four of the isolates were selected for further analysis. To imitate real life scenarios, dried grapes and two types of re-moistened raisins were used. The dried grapes had three different initial water activities (0.54;

0.69; 0.76). The mean FB_2 and FB_4 production in the dried grapes were 91-1747 µg/kg and 9-69 µg/kg, table 10. Fumonisin B_6 was only found in two samples at very low levels. All samples had lower fumonisin levels compared to fresh grapes. The overall trend from this were, that the fumonisin production decreased with decreasing a_w, which correlates with our earlier results, that lower a_w results in a low fumonisin production (Chapter 3.1).

Table 10: Production of fumonisins by *A. niger* in dried grapes after 7 days growth at 25˚C.[A](PAPER 2)

Isolate	Weight loss %	Initial a_w	Fumonisin B_2 µg/kg	Fumonisin B_4 µg/kg[B]	Fumonisin B_6 µg/kg[C]	Ochratoxin A µg/kg[D]
28753	32	0.76±0.04	1747±316	48±22	n.d.	n.d.
	38	0.69±0.01	1128±329	48±16	8±2	n.d.
	52	0.54±0.09	91±73	9±4	n.d.	n.d.
28934	32	0.76±0.04	1363±954	69±51	7±5	n.d.
	38	0.69±0.01	1033±460	50±23	n.d.	n.d.
	52	0.54±0.09	636±397	24±16	n.d.	n.d.
28948	32	0.76±0.04	313±323	54±47	n.d.	n.d.
	38	0.69±0.01	154±71	27±11	n.d.	n.d.
	52	0.54±0.09	144±104	29±19	n.d.	n.d.
29019	32	0.76±0.04	203±106	25±11	n.d.	n.d.
	38	0.69±0.01	214±137	28±13	n.d.	n.d.
	52	0.54±0.09	102±76	10±5	n.d.	n.d.

[A]: The weight loss was 32% (4 h), 38% (5 h), and 52% (6.5 h). The values are means of the quadruplicates, plus/minus the standard deviation, LOD: 5 µg/kg. [B]: Assuming same response factor as fumonisin B_2. [C]: Assuming same response factor as fumonisin B_1.. [D]: LOD: 3 µg/kg. nd: not detected.

Two types of re-moistened raisins were made each with different initial a_w (0.77;0.95), both types initially surface sterilized. **Scenario 1**: Raisins were placed on a filter placed on water agar, allowing the raisin to be kept at stagnate or increasing a_w, figure 6. **Scenario 2**: Raisins were covered with water and kept overnight at 5 °C and placed in an empty Petri dish, the raisin were hereby kept in an environment with decreasing a_w, figure 6.

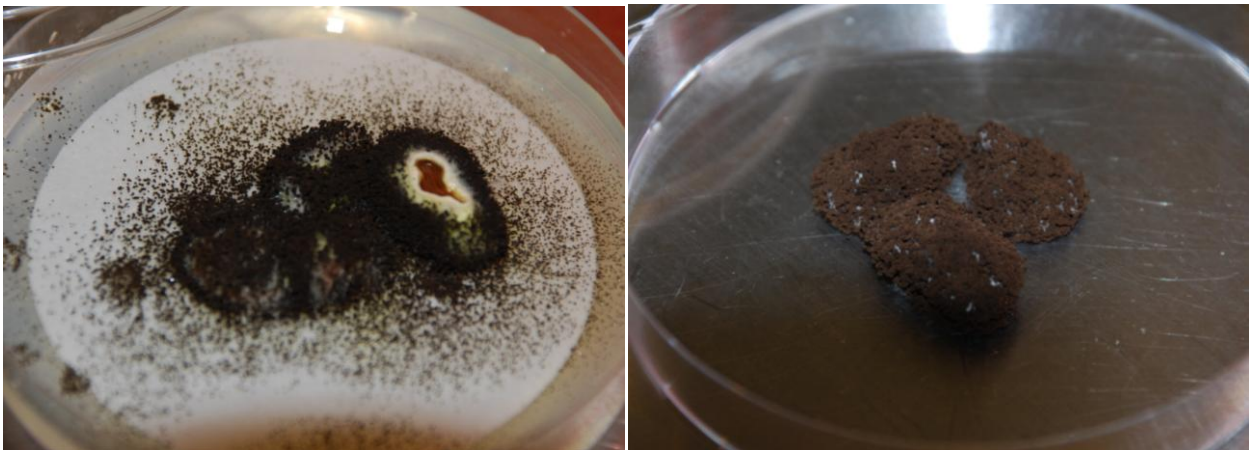


Figure 6: Two types of worst-case scenarios of re-moistened raisins. Left: Re-moistened raisins were placed on a filter on top of the water agar. Right: Raisins were covered with water and kept overnight at 5 °C and placed in an empty Petri dish

Fumonisin production in all samples was markedly higher in scenario 1 (229-6476 µg/kg) than scenario 2 (5-784 µg/kg), table 11. Only two strains produced FB_6 (9-68 µg/kg) at low levels compared to FB_2 and FB_4.

Table 11: Production of fumonisins by *A. niger* in grapes after 7 days growth at 25˚C[A]. (PAPER 2)

Isolate IBT	Weight gain	Initial a_w	Fumonisin B_2 µg/kg	Fumonisin B_4 µg/kg[B]	Fumonisin B_6 µg/kg[C]	Ochratoxin A µg/kg[D]
28753	7% (agar)	0.77±0.05	1160±686	105±56	22±12	n.d.
	50% (water)	0.95±0.001	407±249	47±25	7±5	n.d.
28934	7% (agar)	0.77±0.05	229±58	27±7	n.d.	n.d.
	50% (water)	0.95±0.001	5±5	n.d.	n.d.	n.d.
28948	7% (agar)	0.77±0.05	459±430	45±31	n.d.	n.d.
	50% (water)	0.95±0.001	112±51	12±4	n.d.	n.d.
29019	7% (agar)	0.77±0.05	6476±1139	356±46	68±12	n.d.
	50% (water)	0.95±0.001	784±636	672±422	9±5	n.d.

[A]: The values are means of the quadruplicates, plus/minus the standard deviation, LOD: 5 µg/kg.
[B]: Assuming same response factor as fumonisin B_2.
[C]: Assuming same response factor as fumonisin B_1.
[D]: LOD: 3 µg/kg. nd: not detected

Since *A. niger* is able to produce fumonisins in grapes, the derived products needed to be looked into, especially because of the previously reported problems with ochratoxin A in wine and raisins[28,174,178-180], as well as the findings of FB_2 in grape must[137]. As a result, it was decided to investigate retail raisins. Totally, 21 raisin brands were screened for fumonisins. FB_2 and FB_4 were detected in 10 samples. The detected levels were FB_2: 1.3 -13 µg/kg and FB_4: 0.31-1.3 µg/kg, table 12. Of the 10 positive samples 7 originated from California, 2 from Chile and 1 from Turkey.

Table 12: Overview of the positive raisin samples and their average fumonisin content[a]. (PAPER 4)

Brand	Origin	Grape variety	Organic	Fumonisin B_2 (µg/kg)	Fumonisin B_4 (µg/kg)[b]
1	California	Thompsons Seedless		4.9±0.4 4.5±1 13.0±1 7.5±0.8	0.26±0.05 0.8±0.4 1.3±0.4 0.9±0.2
2	California	Thompsons Seedless		2.7±0.2	0.29±0.1
3	California	Thompsons Seedless	√	1.8±0.2	<LOQ[c]
4	California	Thompsons Seedless	√	2.6±0.4	0.83±0.5
5	California	Thompsons Seedless		3.8±0.6	0.31±0.09
6	California	Thompsons Seedless		4.1±0.6	1.0±0.1
7	California	Thompsons Seedless	√	1.3±0.6	0.25±0.2
12	Turkey	Sultana		4.8±2	0.75±0.1
20	Chile	Golden		<LOQ	ND
21	Chile	Golden		1.4±0.6	<LOQ

[a] Fumonisin concentration is given as the average of three determinations from the same package, ±SD.
[b] Fumonisin B_4 was calculated assuming a 4 fold better response factor than for fumonisins B_2 shown at one spiking experiment. Fumonsin B_1 and B_6 were not detected in any of the packages.
[c] LOQ, limit of quantification: fumonisin B_2: 1 µg/kg and for B_4 estimated to 0.25 µg/kg.
[c] ND, not detected, limit of detection for fumonisin B_2 was 0.3 µg/kg and for B_4 0.1 µg/kg

To further investigate the magnitude of the fumonisin contamination in grape derived products, we chose to look at wine, and since there, at that point, was no method for detection of fumonisins in wine, a prime objective for this project was development of a method. For this purpose several SPE columns were tried. Initially, SAX was the choice, however irreproducibility and poor recovery urged for a different approach. Instead cation-exchange was attempted, targeting the amine group of the B-series fumonisin. Testing of six different cartridges (mixed-mode and polymeric), Strata-X-C and OASIS MCX was found to be superior to the others. Due to a slightly better reproducibility Strata X-C were chosen. The method was validated showing a mean recovery of 83% with an RSD of 12%. Using this method, 77 wine samples were screened for fumonisin content, and 23% of these were positive (n=18), with fumonisin levels between 1-25 µg/L, table 13. The frequency and levels were similar to a contemporary study of Italian wines[114], which showed a positive fraction of 17% and fumonisin levels between 0.4-2.4 µg/L.

Table 13: Overview of the positive wine samples[a]. (PAPER 3)

Sample number	Country	Grape sort	Winetype	Year	Fumonisin B_2 (µg/L)
10	Australia	Shiraz	Red	2008	1.0
24	France	Carignan, Grenache, Syrah	Red	2003	5.1
37	France	Grenache, Syrah	Red	2003	1.3
68	France	Grenache, Syrah, Mourvedre	Red	2008	6.5
9	Italy	Merlot	Red	2007	7.0
14	Italy	Corvina, Rondinella, Molinarea, Negrana	Red	2007	6.7
39	Italy	Montepulciano, Aglianico	Red	2005	2.1
40	Italy	Merlot, Corbina	Red	2007	3.9
58	Italy	Negroamaro, Primitivo	Red	2006	1.6
63	Italy	n.s.	Red	2007	1.2
66	Italy	Corvina, Rondinello, Molinara	Red	2004	3.2
89	Italy	n.s.	White	2008	1.0
104	Portugal	n.s.	Port	n.s.	2.8
7	Romania	Pinot Noir	Red	2006	20
5	South Africa	Cabernet Sauvignon	Red	2007	2.4
2	Spain	Monastrell	Red	n.s.	2.1
27	Spain	Tempramito, Gaunacha	Red	2005	1.9
33	USA, CA	Zinfandel	Red	1998	25

[a]The description of the wines was read off the label of the bottle
n.s.: Not specified

Of the 18 positive samples, 16 were red, one white and one port wine. A similar high frequency of mycotoxin in red wine is also seen with ochratoxin A contamination, which is probably caused by the differences in winemaking[175,178,181]. Furthermore, results indicated, although not significantly, that Italian wine (35%) was more frequently contaminated compared to Spanish (22%) and French (14%) wine, although at lower average levels than French but higher than the Spanish.

The levels found in the raisins (1.4-14.3 µg/kg) and wine (1-25 µg/L) as well as those found in coffee beans[36] (1-9.7 µg/kg) are comparable; however, the frequency of contaminated samples are more than twice as large in raisins and coffee beans than wine (23 %) with 48 % and 58 %, respectively. On the basis of the tested wine and raisin samples, it can be concluded that the frequency of heavily infected grapes is low, since the detected levels are 100-400 times lower than those found in highly contaminated grapes in this study, and a prior by Logrieco *et al.*[137]. This could be due to efficient removal of infected/damaged berries, initiated after problems with ochratoxin A in grapes and derived products were reported in the late 1990s[178,182,183].

The detection of FB_4 in retail raisins agree with the previous results from artificially infected grapes and raisins; however, fumonisin B_4 was not identified in the studies of fumonisins in wine[114] and grape must[137]. During raisin production there is the possibility of both pre- and post-harvest contamination, since the grapes are often sun-dried[184]. The reason for the FB_4 production, could

be caused by a different growth physiology and metabolism of *A. niger* during the drying process of the grapes. Which is probably caused by a high growth rate and as a consequence, some of the FB_4 is not converted into FB_2, before the water activity decreases below *A. niger* growth limits (~0.85)[185]. The postharvest growth together with the water loss during the drying process of the vine fruits and the simultaneous concentration of the fumonisins, may explain why fumonisin B_4 is not detected in this, and other wine studies[137].

3.2.2 Fumonisin from *Aspergillus niger* in other food commodities

Besides grape, wine, and raisins where *A. niger* is a considered huge problem, other commodities where *A. niger* could pose a risk, were also looked at. Black tea/puerh tea and peanut/peanut butter were chosen. The puerh tea was chosen because it was reportedly fermented with *A. niger,* which also is the dominant organism in black tea[186-190]. Ten teas were investigated, five regular black tea and five puerh tea. Incubation of the teas on DG18 led to the isolation of 47 black Aspergilli. To determine their metabolite profile including the potential fumonisin production, the strains were analyzed by LC-MS and HPLC-UV. None of the 47 strains had detectable amounts of fumonisins or ochratoxin A, but other metabolites were identified. All strains produced asperazine with 12 furthermore producing antafumicin, consequently none of the strains could be *A. niger*, since these compounds are not associated with *A. niger*[144]. Production of antafumicin is only found in *A. acidus*, and thus the 12 strains could be characterized as this species. From their metabolite profile the remaining 35 strains were provisionally identified as *A. acidus* or *A. tubingensis*. Sequences of a part of the calmodulin gene in 17 of these strains, were thus examined. The partial sequences were identical to that of *A. acidus*, and all 35 strains were hereby identified as *A. acidus*. Since all black Aspergilli were identified as *A. acidus*, no obvious risk of mycotoxin contamination would be associated with black and puerh tea. The suggested non-toxicity is supported by a toxicity study, in which no adverse effects, were observed after intake of puerh tea extracts[191].

An additional food commodity was investigated; this was peanut and peanut butter. The potential for fumonisin production in peanuts were determined by worst case scenarios by inoculation of *A. niger* on shelled peanuts, figure 7.

Figure 7: Examples of worst-case scenarios of *A. niger* contaminated peanuts after 7 days growth at 25°C

Four *A. niger* strains were inoculated on peanuts and incubated for 7-14 days at 25 °C. The mean production of FB_2 varied more than 40 fold from 0.22 mg/kg (IBT 29019) to 9.4 mg/kg (IBT 18741). The measured fumonisin concentrations (table 14) are similar and comparable to those previously detected in grapes and raisins (0.2-8 mg/kg)[137] (Chapter 3.2.1).

Table 14: Production of fumonisins by *Aspergillus niger* in peanuts after 7-14 days growth at 25 °C

Incubation period	IBT 18741 (FB_2, mg/kg)		IBT 28753 (FB_2, mg/kg)		IBT 29019 (FB_2, mg/kg)		IBT 29331 (FB_2, mg/kg)	
	#1	#2	#1	#2	#1	#2	#1	#2
7 days	5.8	4.8	3.5	1.6	0.43	0.014	2.5	1.0
14 days	9.6	9.1	2.5	3.9	0.56	3.4	3.9	7.6

Since *A. niger* is able to produce fumonisins when growing on peanuts, the potential for fumonisin contamination was present and peanut retail products were therefore screened. Analysis of 15 peanut samples (from local markets in Thailand) and nine peanut butter brands (peanuts originating from four countries) all analyzed in triplicate showed no indications of fumonisins (LOD: 0.004 mg/kg). Despite *A. niger* is a common contaminant of peanuts[143,192-196], and that when grown on the peanuts, fumonisins are produced in high quantities (up to 2000 higher than our detection limit). We assume that the combination of: i) extensive damage to the nuts made

by *A. niger* (in some cases they are heavily digested), and: ii) the high attention to reduce aflatoxin exposure[24] effectively removes *A. niger* infected peanuts and thus fumonisins.

3.3 Single kernel analysis of fumonisins from *Fusarium* in maize

The main crop where fumonisins are a concern is maize. Several reports from around the world, have described fumonisin contamination of maize and derived products. A new approach was initiated within this thoroughly investigated area, by investigating single kernels, instead of the traditionally approach, where the average content in lots was determined. Using this approach, the aim was to clarify, the effect of manual visual sorting, as well as the content and distribution of fumonisins in the single kernels. The maize was collected from subsistence farmers in South Africa, who had classified the maize in two categories: “Good” and “Moldy”. In total ten batches were investigated, five from each category.

The infection rate of the ten batches was determined by random selection of 100 kernels and counting of infected/damaged kernels. Good quality batches had infection rates below 3%, while the moldy batches had a marked difference, with infection rates of 6-19%, table 15, p29. To survey the mycobiota of the maize, visibly infected/damaged kernels were placed on four different media (24 per batch, n_{total}=240) and strains were isolated after 7 days growth at 25 °C. *Fusarium* infections were seen in 17-83 % of the kernels by primarily *F. verticillioides* and *F. subglutinans*. These findings were in agreement with previous studies[197,198]. Other species found were *Penicillium aurantiogriseum*, *P. brevicompactum, P. concavorugulosum, P. crustosum*, *P. pittii*, and *Aspergillus wentii*.

Analysis by LC-MS/MS of infected/damaged (n=300) and uninfected/undamaged (n=100) kernels (figure 8) for fumonisin contamination, showed that 15% had detectable amounts of fumonisins and 4% were considered highly infected, because the fumonisin levels were above 100 mg/kg. None of the visually uninfected/undamaged kernels contained fumonisins. However, in the infected/damaged kernels fumonisin levels were batch dependent and varied from 3-40% with a total fumonisins (FB_1, FB_2, FB_3, and FB_4) range of 1.8-1428 mg/kg. Even though low-quality kernels were more frequently found in the poor-quality maize, low quality kernels from the good-quality maize had generally higher fumonisin content than those from the moldy batches.

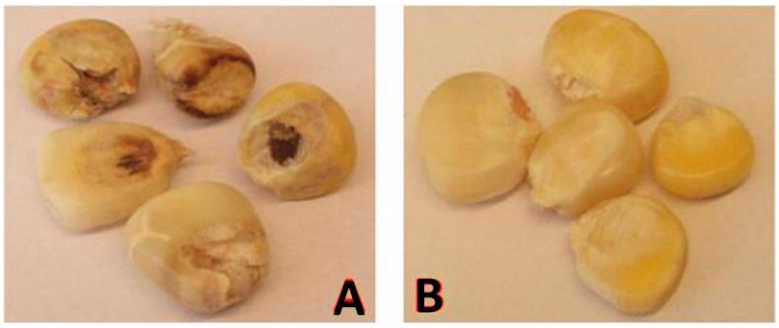


Figure 8: Infected/damaged (A) and uninfected/undamaged (B) kernels

The fumonisin content in the batches were estimated using infection rate, average kernel weight, batch weight, and average fumonisin content. Good quality batches had an average estimated fumonisin content of 0.28-1.1 mg/kg and moldy batches contained 0.03-6.2 mg/kg. Two of the batches exceeded the maximum guidance level of 4 mg/kg in maize for human consumption recommended by the USFDA[21] and four exceeded the European union regulatory limit of 1 mg/kg[37].

Since only 15 % of the 400 kernels were positive for fumonisins, 80 samples were send to Austria for a multi-mycotoxin screen[199] to identify toxins from the non-fumonisin producers. The 80 samples were selected to represent a combination of high, low, and no amount of fumonisins. Overall, 21 compounds were detected in the 80 kernels; however four kernels did not contain any detectable amounts of the analyzed toxins. Fumonisins were detected in 50 samples, of which one was characterized as uninfected, table 16, p30. Other frequently detected compounds were fusaric acid (n=53), emodin (n=27), chanoclavine (n=25), equisetin (n=14), and fusaproliferin (n=12). Seldom found compounds were agroclavine (n=3), altertoxin-I (n=3), beauvericin (n=7), moniliformin (n=4), nivalenol (n=4), rugulosin (n=4), and tentoxin (n=3). Alternariol, calphostin C, and cyclosporins A, C and H, were also detected, however only in single cases.

Table 15: Infection rate of baches and fumonisin (FB) content in uninfected and infected maize kernels (n_{total}=400).

Batch	416	438	440	447	448	404	429	431	432	455
Quality[a]	Good	Good	Good	Good	Good	Mouldy	Mouldy	Mouldy	Mouldy	Mouldy
Infection rate (%) [b,c]	66	66	83	66	100	100	83	66	83	83
Dominating FB-producing fungi (%) [b,d]	*F.sub(17)*	*F. vert (17), F.sub (17)*	*F. vert (17)*	*F.vert (50)*	*F. sub (33), F.vert (17)*	*F. vert (50), F. sub (33)*	*F. vert (17).*	*F. vert (17)*	*F. sub (33)*	*F. vert (17)*
Other fungi[b,c,d]	*P. conca, A. wentii, P. auran*	*P. conca, P. auran*	*P. conca*	*A. wentii*		*Eurotium sp.*		*P. crust, A. wentii*	*P. pittii, P. brevi*	*P. conca, P. crust*
Visibly infected or damaged (n=100)	2%	3%	3%	3%	3%	6%	14%	7%	19%	12%
Uninfected kernels contaminated with FB (n=10)	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Visually infected kernels contaminated with FB (n=30)	20%	27%	27%	13%	10%	23%	10%	40%	23%	3%
FB contents in infected kernels (min-max, mg/kg)	34.4-1428	4.0-90	1.9-715	1.8-432	5.8-929	2.1-257	39-736	3.2-968	4.6-713	8.37
Mean FB content in infected kernels (mg/kg)[e]	330	49	114	149	328	54.8	297	166	159	8.37
Median FB in infected kernels (mg/kg)	53	42	30	82	50	4.8	117	33	41	8.4
Total FB content (mg/kg)[f]	1.1	0.28	0.79	0.52	0.85	0.67	3.7	4.1	6.2	0.03

Note: Fumonisin content is the total FB_1, FB_2, FB_3 and FB_4 content.

Abbreviations: F. *vert*: *F. verticillioides*; *F. sub*: *F. subglutinans*; *P. auran.*: *P. aurantiogriseum*; *P. brevi*: *P. brevicompactum*; *P. conca*: *P. concavurugulosum*; *P. crust.*: *P. crustosum.*

[a] As sorted by the five subsistence farmers.

[b] Surface sterilization in hypochlorite prior to plating for a total of 180 randomly selected kernels (18 per batch with 6 on DG18%, 6 on CZID, and 6 on PCMA).

[c] Plating of 60 visibly infected or damaged kernels on water agar per batch.

[d] Not including sterile mycelia and species found only once among the 240 kernels.

[e] LC-MS/MS on Ultima QQQ. LOD_{FB1} of 0.16 mg/kg and LOD_{FB2} of 0.057 mg/kg

[f] Total FB content were calculated from the infection rate, mean kernel weight, batch weight, and mean fumonisin concentration.

Table 16: Distribution, mean and likely producer of metabolites found in visual infected and uninfected kernels.

Mycotoxin	% in uninfected kernels (n=8)	Mean in uninfeted kernels	% in infected kernels (n=72)	Mean in infected kernels	Likely producer	Supported by microbiology	Produced pre or post harvest
Alternariol	0	-	1	13.8 µg/kg	*Alternaria*	No	Pre[a]
Altertoxin-I	13	0.26 µg/kg	3	0.18 µg/kg	*Alternaria*	No	Pre[a]
Beauvericin	13	0.32 µg/kg	8	8.4 µg/kg	*F. subglutinans*	Yes	Pre
Calphostin C	0	-	1	58.1 µg/kg	*Cladosporium*	No	Pre[a]
Cyclosporins	13	6.5 mg/kg	0	-	*F. solani* or *Tolypocladium*	No	Pre
Equisetin	25	83 µg/kg	17	75 µg/kg	*F. equiseti*	No	Pre
Fumonisin B_1	0	-	60	27 mg/kg	*F. verticillioides, F. subglutinans*	Yes	Pre
Fumonisin B_2	13	0.0035 mg/kg	65	8.7 mg/kg	*F. verticillioides, F. subglutinans*	Yes	Pre
Fumonisin B_3	0	-	51	2.1 mg/kg	*F. verticillioides, F. subglutinans*	Yes	Pre
Fumonisin B_4	0	-	49	1.6 mg/kg	*F. verticillioides, F. subglutinans*	Yes	Pre
Fusaproliferin	0	-	17	11 µg/kg	*F. subglutinans*	Yes	Pre
Fusaric acid	13	0.2mg/kg	64	34 mg/kg	*F. verticillioides, F. subglutinans* and other related	Yes	Pre
Moniliformin	0	-	6	202 µg/kg	*F. subglutinans*	Yes	Pre
Nivalenol	13	2.01 µg/kg	4	59 µg/kg	*F. equiseti, F. graminearum*	(Yes)[b]	Pre
Tentoxin	13	0.37 µg/kg	3	0.12 µg/kg	*Alternaria*	No	Pre[a]
Agroclavin	13	0.13 µg/kg	3	0.25 µg/kg	*P. concavurugulosum*	Yes	Post
Chanoclavine	38	4.5 µg/kg	31	1.2 µg/kg	*P. concavurugulosum*	Yes	Post
Emodin	75	0.73 µg/kg	29	2.2 µg/kg	*Eurotium, A. wentii*	Yes	Post
Rugulosin	0	-	6	372 µg/kg	*P. concavurugulosum*	Yes	Post

The majority of these compounds are presumably produced pre-harvest derived from the naturally growth of *Alternaria* and *Fusarium* in the field. The remaining part, is assumed to be produced post-harvest, since these are produced by *Penicillium*, *Aspergillus,* and *Eurotium*. Kernels with fusaproliferin and/or moniliformin had often lower amounts of fumonisins, indicating the presence of *F. subglutinans*, which is known as a poor fumonisin producer[200]. Additionally, kernels with both fusaric acid and fumonisins had an average fusaric acid content of 37 mg/kg, compared to 112 mg/kg in kernels only containing fusaric acid. Fusaric acid inhibits plant growth, and is suggested to be antifungal and antinematodic[201,202]. The high concentration in samples without fumonisins, indicate that non-toxigenic Fusaria are present in maize, and supports the possibility of using these fungi as biocontrol agents against fumonisin producing species, as is the case for aflatoxins and *A. flavus*[203-205].

Equisetin is a known metabolite from *F. equiseti*[206], which also produce nivalenol[147]; however, this species was not isolated during the fungal screening. Nivalenol could also originate from other *Fusarium* species like *F. graminearum*[147], although this species was only seen once in the screening of 240 kernels. The domination of *F. subglutinans* and *F. verticillioides* is most likely the cause for suppression of other *Fusarium* species.

Emodin is produced regularly by several *Eurotium* and *A. wentii,* they are common superficial contaminants of dried grain. The finding of these compounds in the visually uninfected kernels, could be caused by superficial growth, which is easy abraded off the kernels, that hereby appear uninfected. Another important mycotoxin in African maize is aflatoxin[207], yet no aflatoxin was detected in either of the kernels, which is an outcome of the absence of *A. flavus*/*A. parasiticus* in the fungal screening.

The low amount of fumonisin positive kernels led to the thought of how the fumonisin concentration would be affected if the poor-quality kernels were sorted out from the batch. This effect were estimated by removal of the 4% highly infected kernels (>100 mg/kg). A 71% reduction in fumonisin levels was estimated, demonstrating that this simple approach is a powerful tool for mycotoxin reduction. The strategy of removing infected kernels was successfully implemented in an intervention study in the same rural area of Transkei[208]. Training of the farmers led to a better result than estimated, giving an 84% reduction with less than 4% weight loss[198,208,209].

3.4 Fumonisin production by other fungal genera

For almost 30 years *Fusarium* was known as the only fumonisin producer, and then came the discovery of a production of FB_2, FB_4 and the newly discovered FB_6 in *A. niger*[34-36]. This small group of fumonisin producing fungi was further expanded during a routine screening of cyclosporine producing fungi with a multi-detection method[199], that led to the discovery that a *Tolypocladium* (figure 9) strain also produced fumonisins. *Tolypocladium* are used in the medical industry for the production of cyclosporines. The cyclosporines are used worldwide as a immunosuppressive drug in organ transplant recipients[210].

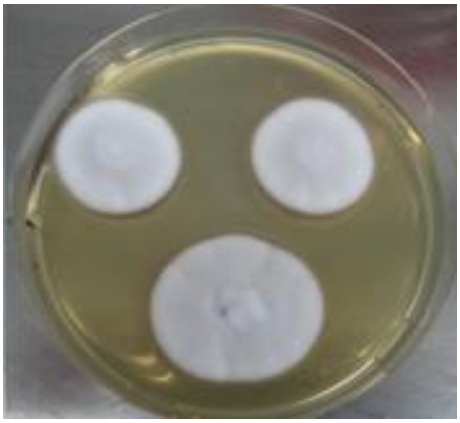


Figure 9: *Tolypocladium inflatum* on YES agar after 7 days growth at 25°C

Due to the important use of *Tolypocladium* species, further investigation of this new exiting discovery was performed. Screening of 12 strains from three different species showed that all 12 produced FB_2, but only ten produced FB_4, table 17. The additional FB's (FB_1, FB_3, FB_6) were not detected in the 12 strains.

Table 17: Production of FB_2 and FB_4 by *Tolypocladium cylindrosporum, T. geodes* and *T. inflatum*

Species	FB_2 positive	FB_4 positive
T. cylindrosporum	3/3	3/3
T. geodes	3/3	1/3
T. inflatum	5/5	5/5

The fumonisin profile of *Tolypocladium* is similar to that of *A. niger,* but significantly different from that of *Fusarium*. This appear to be in agreement with the proposed fumonisin biosynthesis of Butchko *et al*.[215], where FB_2 and FB_4 are products from the same biosynthetic pathway. FB_1 and FB_3 are produced in a different pathway, which indicate that *Tolypocladium,* just as *A. niger*, are at least missing the *FUM*2 gene in the fumonisin gene cluster[215].

To obtain new details on the fumonisin producing *Tolypocladium* species, the effect of media composition and temperature was investigated. Three strains were chosen and inoculated on ten different laboratory media along with an *A. niger* strain as a control organism. The ten media were CYA[166], CYAS [166], dichloran Rose bengal yeast extract sucrose agar (DRYES)[211], potato carrot agar (PCA)[212], malt extract agar (MEA according to Blakeslee)[147], oatmeal agar (OAT)[166], PDA (Difco), V8-juice agar with antibiotics (V8)[212], YES[166], and dichloran 18% glycerol agar (DG18)[213]. On each medium at least one of the *Tolypocladium* strains were able to produce fumonisins, figure

10. Two media, PDA and YES, stood out as the best for all three strains. Levels of FB_4 to FB_2 were in the range of 0-95%; however, mostly between 10-30%. The maximal total fumonisin production (FB_2 + FB_4) of the three strains was 0.072 µg/cm^2 for IBT 41581, 30 µg/cm^2 for IBT 41582, and 1.3 µg/ cm^2 for IBT 41583. These values are similar to the earlier study (Chapter 3.1) with *Fusarium* and *A. niger* and their detected amounts of 0.006-22 µg/cm^2 and 0.27-21 µg/cm^2, respectively.

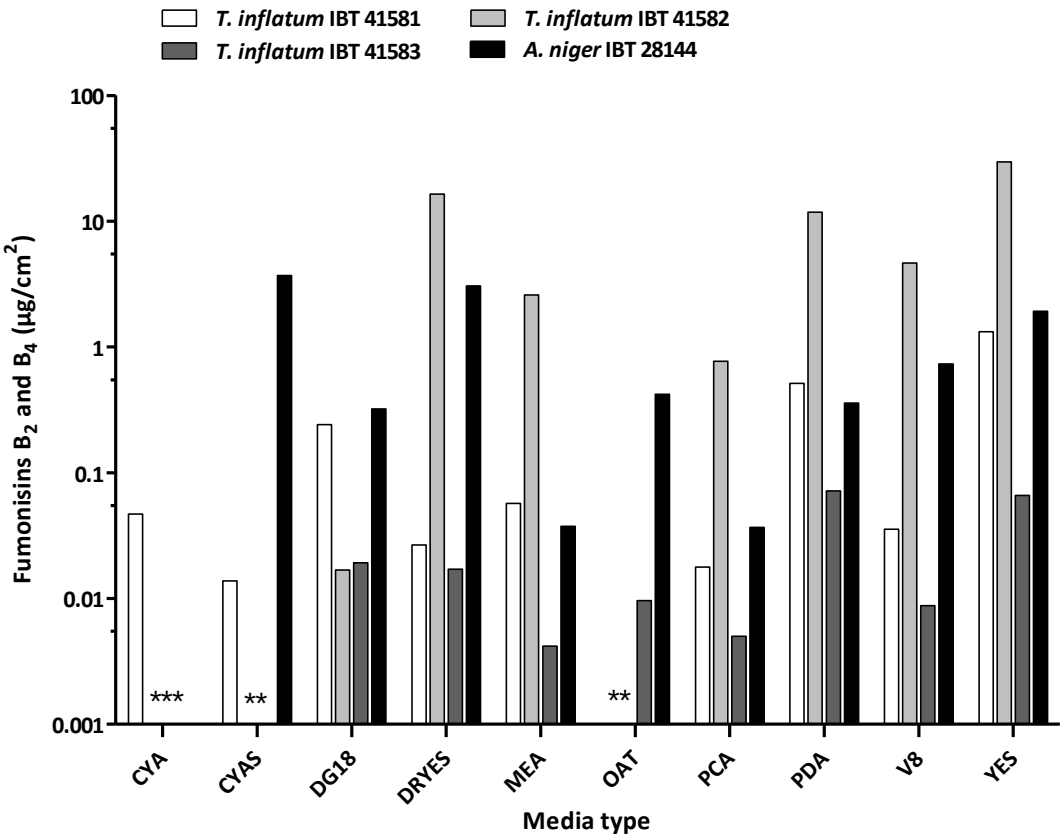


Figure 10: Total production of fumonisin (FB_2 and FB_4) by three *Tolypocladium inflatum* strains (IBT 41581, IBT 41582, IBT 41583) and one *Aspergillus niger* (IBT 28144) on different media at 25°C. *Not detected

To determine further criteria for fumonisin production by *Tolypocladium*, the effect of temperature were studied. Five different temperatures in the range of 15-37 °C were used. Fumonisin production occurred from 15-30 °C, figure 11; samples at 37 °C were not analyzed due to the inability to grow at this temperature. The temperature effect appeared to be strain dependent, since two strains had a maximal production of fumonisins at 30 °C and the last at 25 °C. Even though *Tolypocladium* is not a danger in food, there are possibilities for a fumonisin contamination from this fungus. Not only is it used in the pharmaceutical industry as a producer of cyclosporin A[210], but it has also been suggested as a possible fungal biological control agent, because of the entomopathogenicity of cyclosporines and efrapeptins[214]. The in house certified

cyclosporin A standard was tested, but did not contain any detectable fumonisins. With this new discovery, the amount of reported fumonisin producing organisms is expanded to a total of 31 species, Table 2.

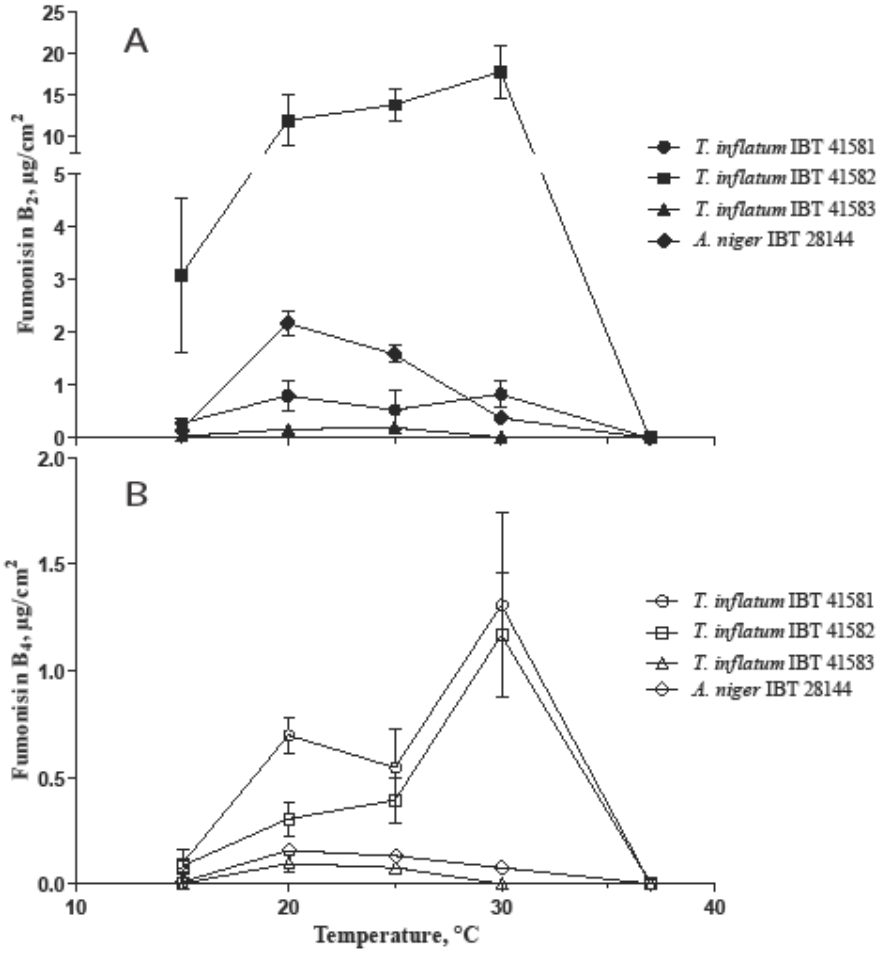


Figure 11: Production of fumonisins B_2 (A) and B_4 (B) by three strains of *Tolypocladium inflatum* (IBT 41581, IBT 41582, IBT 41583) incubated for 7 days at 15, 20, 25, 30 and 37 °C on YES. The standard deviation of triplicates is shown by the error bars. Fumonisin concentration was 0 µg/cm^2 at 37 °C due to lack of growth.

3.5 Genotoxicity studies of fumonisins

Some work during the past three years has been put into the investigation of the genotoxicity of fumonisins, as seen in chapter 2. The overall idea of this project was to purify FB_2, FB_4, and FB_6 from *A. niger* and compare the genotoxicity of these compounds to FB_1 and FB_2 from *Fusarium*. Since the purification process was difficult to carry out, only a limited amount of preliminary work was performed. The assay of choice, was the comet assay, also called single cell gel electrophoresis. This assay is widely regarded as a quick and reliable method for analysis of DNA

damage in individual cells[216]. Optimization of the assay, as well as preliminary testing of fumonisins was carried out. Fumonisins were tested against two cell lines, HepG2 (liver) and Caco-2 (colon). Fumonisin B_1 and FB_2 from *Fusarium* were only tested in single replicates at concentrations up to 588 µg/ml, with no signs of either cytotoxicity or genotoxicity; however, these results and replicates are too few to make any firm conclusions on. It is noteworthy that available information of the genotoxic properties of FB_1 is limited and controversial due to conflicting results[31,217]. This sub-project stalled, since the purification process of fumonisins from *A. niger* was problematic, often resulting in methylated fumonisins, but the purification is still ongoing. Next step in this, is the making of a mutant with an over-expression of the *FUM* genes.

3.6 Overall discussion

Aspergillus niger produced fumonisins in every single tested commodity, raisin, grapes, and peanuts. However the reported fumonisin levels in the food and feed from the past three years are all within the range of 1-25 µg/kg, with one exception of 400µg/kg in a grape must sample[36,113,114,137,218]. The levels in raisins reported by Varga *et al.* appear to be doubtful due to the high levels and the reported FB_1 and FB_3 production[136], it is furthermore disputed[219,220] and is therefore excluded from this comparison. The levels of fumonisins are significantly lower than those produced in worst-case scenarios, indicating that fumonisins from *A. niger* are not a significant problem, since the highest concentration found is 10-160 times (omitting the grape must sample) below the regulatory limit for fumonisins in maize and maize products set by EU[37]. This is most likely due to the enormous effort put into reducing the already know problem, with ochratoxin A in similar commodities. The visual screening in the field as well as mechanical sorting applications like color sorters[24], has significantly reduced the mycotoxin contamination. Multiple washing steps are also implemented in the production of certain food, e.g. raisins[184]; washing crops could also be the cause for the low contamination since this procedure have previously shown to reduce the mycotoxin concentration[198,221]. Furthermore, implementation of “Good Agricultural Practice” and “Code of Practice” from FAO/WHO[222] and similar organizations is also likely a liable reason for the significant impact within prevention and reduction of mycotoxin contamination in food and feeds.

Due to the status as an industrial workhorse, production of fumonisins by *A. niger* in an industrial setting, is essential to investigate. Three industrial strains (NRRL 3= IBT 23539; NRRL 567=IBT 26387, NRRL 2001=IBT 26392) used in this study have previously been used for production of organic acids and extracellular enzymes[223-226]. All three strains produced fumonisins on solid media under all conditions, except high temperature (>37 °C). A recent report mimicked industrial citric acid production conditions and tested *A. niger* production of fumonisins and ochratoxin A. In most cases fumonisins as well as ochratoxin were produced[146]. In this context, it should be mentioned that industrial production companies, presumably have knocked the fumonisin gene cluster out of their *A. niger* production strains. If this is not the case, fumonisin production could likely occur during industrial conditions, and monitoring industrial end products and/or waste being used as feed for domestic animals[227,228] is essential. Yet, it should be mentioned that batches of all industrial products are tested for cytotoxicity, carcinogenicity, and similar diverse effects before product approval. These tests would have indicated if ochratoxins and/or fumonisins are produced, but no reports of this have been published[154].

Preliminary results from the genotoxicity study reveal that FB_1 and FB_2 from *Fusarium* is not genotoxic, in concordance with other studies[229,230]. However fumonisins from *A. niger* is not tested yet, and this could change the picture completely due to e.g. stereochemistry. Because several genes are not present in the fumonisin gene cluster compared to that of *Fusarium*[55,56], a different biosynthesis could occur and hereby cause differences in the stereochemistry. An example of the vital role of stereochemistry is thalidomide, where small changes in the stereo chemistry made a harmless compound active [231]. Currently, there are strong indications that the stereochemistry of FB_2 originating from *Fusarium* and FB_2 from *A. niger* is similar[35]. Additionally, the toxicity of FB_4 and FB_6 has never been tested, even though they are produced to a lesser extinct than FB_2, they could still be more potent. Toxicity combinations of mycotoxins are also imperative to investigate, since mycotoxins most likely are present in combinations, rather than alone. With the discovery of a fumonisin production in *A. niger* the combination of ochratoxin and fumonisins is even more relevant, especially because more than 60 % of ochratoxin A producing *A. niger* also produces fumonisins[146].

4 Conclusion

The frequency of fumonisin producers within the *A. niger* species is approx. 75%, and fumonisins are produced in grapes, raisins, and peanuts at high levels. Although, the frequency and level of fumonisins in retail products are significantly lower. Grapes appear to be the most central commodity for the fumonisin production, affecting the derived products raisins and wine where fumonisins produced by *A. niger* could be detected in 48 % and 23 % of the investigated retail products.

The overall objective of the project is to determine if fumonisins from *A. niger* is a health risk. On the basis of this thesis, there is no evidence that suggest that fumonisins from *A. niger* constitutes a health risk. Especially compared to fumonisins produced by *Fusarium* in maize, since they are found at considerably higher levels.

There is still a small uncertainty, since the toxicity of FB_2, FB_4 and FB_6 from *A. niger* is yet to be evaluated. If the toxicity is similar to fumonisins from *Fusarium*, the significance of this fungi/mycotoxin combination is low, due to the low almost insignificant amounts of fumonisins from *A. niger* in retail products. But at the present moment fumonisins from *A. niger* should not be considered a health risk.

5 References

1. K.N.Daly Roman Mythology A to Z*;* 3rd edition ed.; Chelsea House: New York, 2009.
2. Bennett, J. W. Mycotoxins, mycotoxicoses, mycotoxicology and mycopathologia. *Mycopathologia*. **1987,** *100*:3-5.
3. Dutton, M. F. Fumonisins, mycotoxins of increasing importance: Their nature and their effects. *Pharmacol Ther*. **1996,** *70*:137-161.
4. Bennett, J. W.; Klich, M. Mycotoxins. *Clin Microbiol Rev*. **2003,** *16*:497-516.
5. Bhat, R.; Rai, R. V.; Karim, A. Mycotoxins in food and feed: Present status and future concerns. *Compr Rev Food Sci F*. **2010,** *9*:57-81.
6. Etzel, R. A. Mycotoxins. *JAMA J Am Med Assoc*. **2002,** *287*:425-427.
7. Murphy, P. A.; Hendrich, S.; Landgren, C.; Bryant, C. M. Food mycotoxins: An update. *J Food Sci*. **2006,** *71*:R51-R65.
8. Shephard, G. S. Determination of mycotoxins in human foods. *Chem Soc Rev*. **2008,** *37*:2468-2477.
9. Council for Agricultural Science and Technology Mycotoxins: Economic and health risks*;* Task Force Report No. 116 ed.; Iowa, 1989.
10. Ramos, A.; Sanchis, V.; Marin, S. The prehistory of mycotoxins: related cases from ancient times to the discovery of aflatoxins. *World Mycotoxin J*. **2011,** *4*:101-112.
11. Richard, J. L. Discovery of aflatoxins and significant historical features. *Toxin Rev*. **2008,** *27*:171-201.
12. Schoental, R. Corner of history - Moses and mycotoxins. *Prev Med*. **1980,** *9*:159-161.
13. Lee, M. R. The history of ergot of rye (*Claviceps purpurea*) I: from antiquity to 1900. *The journal of the Royal College of Physicians of Edinburgh*. **2009,** *39*:179-184.
14. Schoental, R. Mycotoxins in food and the plague of Athens. *J Nutr Med*. **1994,** *4*:83-85.
15. Joffe, A. Z. Biological properties of some toxic fungi isolated from overwintered cereals. *Mycopathologia*. **1962,** *16*:201-221.
16. Sargeant, K.; Sheridan, A.; Okelly, J. Toxicity associated with certain samples of groundnuts - Reply. *Nature*. **1961,** *192*:1096-1097.
17. Blount, W. P. Turkey "X" disease. *J Brit Turkey Fed*. **1961,** *9*:52-61.

18. Asplin, F. D.; Carnaghan, R. B. A. The toxicity of certain groundnut meals forpouktry with special reference to their effect on ducklings and chickens. *Vet Rec*. **1961,** *3*:1215-1218.

19. Nesbitt, B. F.; Okelly, J.; Sheridan, A.; Sargeant, K. Toxic metabolites of *Aspergillus flavus*. *Nature*. **1962,** *195*:1062-1063.

20. Sweeney, M. J.; Dobson, A. D. W. Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. *Int J Food Microbiol*. **1998,** *43*:141-158.

21. Food and Agricultural Organization Worldwide regulations for mycotoxins in food and feed in 2003*;* FAO Food and Nutrition Paper No. 81 ed.; FAO: Rome, 2004.

22. Peraica, M.; Radic, B.; Lucic, A.; Pavlovic, M. Toxic effects of mycotoxins in humans. *B World Health Organ*. **1999,** *77*:754-766.

23. Reddy, K.; Salleh, B.; Saad, B.; Abbas, H.; Abel, C.; Shier, W. An overview of mycotoxin contamination in foods and its implications for human health. *Toxin Rev*. **2010,** *29*:3-26.

24. Pitt, J., I; Hocking, A. D. Mycotoxins in Australia: biocontrol of aflatoxin in peanuts. *Mycopathologia*. **2006,** *162*:233-243.

25. EU. Setting maximum levels for certain contaminants in foodstuffs as regards aflatoxin. *Comm Eur Communities*. **2010,** *165*:8-12.

26. van Walbeek, W.; Scott, P. M.; Harwig, J.; Lawrence, J. W. *Penicillium viridicatum* Westling - A new source of ochratoxin A. *Can J Microbiol*. **1969,** *15*:1281-&.

27. van der Merve, K. J.; Steyn, P. S.; Fourie, L.; Scott, D. B.; Theron, J. J. Ochratoxin A a toxic metabolite produced by *Aspergillus ochraceus* wilh. *Nature*. **1965,** *205*:1112-&.

28. EU. EU rules on ochratoxin A extended to coffee, wine and grape juice. *European Commission press release*. **2004,** *IP/04/1215*.

29. Bayman, P.; Baker, J. L. Ochratoxins: A global perspective. *Mycopathologia*. **2006,** *162*:215-223.

30. Richard, J. L. Some major mycotoxins and their mycotoxicoses - An overview. *Int J Food Microbiol*. **2007,** *119*:3-10.

31. Bolger, M.; Coker, R. D.; DiNovi, M.; Gelderblom, W. C.; Olsen, M.; Paster, N.; Riley, R. T.; Shepard, G. S.; Speijers, G. J. A. Fumonisins. In *Safety evaluation of certain mycotoxins in foods.* FAO Food and Nutrition Paper 74. World Health Organization. Geneva. 2001; pp 103-279.

32. Rheeder, J. P.; Marasas, W. F. O.; Vismer, H. F. Production of fumonisin analogs by *Fusarium* species. *Appl Environ Microbiol*. **2002,** *68*:2101-2105.

33. Bezuidenhout, S. C.; Gelderblom, W. C. A.; Gorst-Allman, C. P.; Horak, R. M.; Marasas, W. F. O.; Spiteller, G.; Vleggaar, R. Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. *J Chem Soc Chem Commun*. **1988,** *11*:743-745.

34. Frisvad, J. C.; Smedsgaard, J.; Samson, R. A.; Larsen, T. O.; Thrane, U. Fumonisin B_2 production by *Aspergillus niger*. *J Agric Food Chem*. **2007,** *55*:9727-9732.

35. Månsson, M.; Klejnstrup, M. L.; Phipps, R. K.; Nielsen, K. F.; Frisvad, J. C.; Gotfredsen, C. H.; Larsen, T. O. Isolation and NMR characterization of fumonisin B_2 and a new fumonisin B_6 from *Aspergillus niger*. *J Agric Food Chem*. **2010,** *58*:949-953.

36. Noonim, P.; Mahakarnchanakul, W.; Nielsen, K. F.; Frisvad, J. C.; Samson, R. A. Fumonisin B_2 production by *Aspergillus niger* from Thai coffee beans. *Food Addit Contam A*. **2009,** *26*:94-100.

37. EU. Setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. *Comm Eur Communities*. **2007,** *1126*:14-16.

38. U.S.Food and Drug Administration Guidance for Industry: Fumonisin levels in human foods and animal feeds. *United States Food and Drug Administration, Washington DC*. **2001**.

39. Klich, M. A. Health effects of *Aspergillus* in food and air. *Toxicol Ind Health*. **2009,** *25*:657-667.

40. EU. Setting maximum levels for certain contaminants in foodstuffs. *Comm Eur Communities*. **2006,** *1881*:1-26.

41. Seo, J. A.; Kim, J. C.; Lee, Y. W. Isolation and characterization of two new type C fumonisins produced by *Fusarium oxysporum*. *J Nat Prod*. **1996,** *59*:1003-1005.

42. Musser, S. M.; Gay, M. L.; Mazzola, E. P.; Plattner, R. D. Identification of a new series of fumonisins containing 3-hydroxypyridine. *J Nat Prod*. **1996,** *59*:970-972.

43. Thiel, P. G.; Marasas, W. F. O.; Sydenham, E. W.; Shephard, G. S.; Gelderblom, W. C. A.; Nieuwenhuis, J. J. Survey of fumonisin production by *Fusarium s*pecies. *Appl Environ Microbiol*. **1991,** *57*:1089-1093.

44. Marasas, W. F. O. Discovery and occurrence of the fumonisins: A historical perspective. *Environ Health Perspect*. **2001,** *109*:239-243.

45. Wilson, B. J.; Maronpot, R. R. Causative fungus agent of leucoencephalomalacia in equine animals. *Vet Rec*. **1971,** *88*:484-&.

46. Kriek, N. P. J.; Marasas, W. F. O.; Thiel, P. G. Hepatotoxicity and cardiotoxicity of *Fusarium verticillioides* (*Fusarium moniliforme*) isolates from Southern African maize. *Food Cosmet Toxicol*. **1981,** *19*:447-456.

47. Kriek, N. P. J.; Kellerman, T. S.; Marasas, W. F. O. A comparative-study of the toxicity of *Fusarium verticillioides* (=*F. moniliforme*) to horses, primates, pigs, sheep and rats. *Onderstepoort J Vet Res*. **1981,** *48*:129-131.

48. Kpodo, K.; Thrane, U.; Hald, B. Fusaria and fumonisins in maize from Ghana and their co-occurrence with aflatoxins. *Int J Food Microbiol*. **2000,** *61*:147-157.

49. Branham, B. E.; Plattner, R. D. Isolation and characterization of a new fumonisin from liquid cultures of *Fusarium moniliforme*. *J Nat Prod*. **1993,** *56*:1630-1633.

50. Bartok, T.; Szecsi, A.; Szekeres, A.; Mesterhazy, A.; Bartok, M. Detection of new fumonisin mycotoxins and fumonisin-like compounds by reversed-phase high-performance liquid chromatography/electrospray ionization ion trap mass spectrometry. *Rapid Commun Mass Spectrom*. **2006,** *20*:2447-2462.

51. Nelson, P. E.; Plattner, R. D.; Shackelford, D. D.; Desjardins, A. E. Fumonisin B_1 production by *Fusarium s*pecies other than *F. moniliforme* in Section *Liseola* and by some related species. *Appl Environ Microbiol*. **1992,** *58*:984-989.

52. Chen, J. P.; Mirocha, C. J.; Xie, W. P.; Hogge, L.; Olson, D. Production of the mycotoxin fumonisin B_1 by *Alternaria alternata* f sp *lycopersici*. *Appl Environ Microbiol*. **1992,** *58*:3928-3931.

53. Caldas, E. D.; Jones, A. D.; Ward, B.; Winter, C. K.; Gilchrist, D. G. Structural characterization of 3 new AAL toxins produced by *Alternaria alternata* f sp *lycopersici*. *J Agric Food Chem*. **1994,** *42*:327-333.

54. Solfrizzo, M.; De Girolamo, A.; Vitti, C.; Tylkowska, K.; Grabarkiewicz-Szczesna, J.; Szopinska, D.; Dorna, H. Toxigenic profile of *Alternaria alternata* and *Alternaria radicina* occurring on umbelliferous plants. *Food Addit Contam A*. **2005,** *22*:302-308.

55. Baker, S. E. *Aspergillus niger* genomics: Past, present and into the future. *Med Mycol*. **2006,** *44*:S17-S21.

56. Pel, H. J.; de Winde, J. H.; Archer, D. B.; Dyer, P. S.; Hofmann, G.; Schaap, P. J.; Turner, G.; de Vries, R. P.; Albang, R.; Albermann, K.; Andersen, M. R.; Bendtsen, J. D.; Benen, J. A. E.; van den Berg, M.; Breestraat, S.; Caddick, M. X.; Contreras, R.; Cornell, M.; Coutinho, P. M.; Danchin, E. G. J.; Debets, A. J. M.; Dekker, P.; van Dijck, P. W. M.; van Dijk, A.; Dijkhuizen, L.; Driessen, A. J. M.; d'Enfert, C.; Geysens, S.; Goosen, C.; Groot, G. S. P.; de Groot, P. W. J.; Guillemette, T.; Henrissat, B.; Herweijer, M.; van den Hombergh, J. P. T. W.; van den Hondel, C. A. M. J.; van der Heijden, R. T. J. M.; van der Kaaij, R. M.; Klis, F. M.; Kools, H. J.; Kubicek, C. P.; van Kuyk, P. A.; Lauber, J.; Lu, X.; van der Maarel, M. J. E. C.; Meulenberg, R.; Menke, H.; Mortimer, M. A.; Nielsen, J.; Oliver, S. G.; Olsthoorn, M.; Pal, K.; van Peij, N. N. M. E.; Ram, A. F. J.; Rinas, U.; Roubos, J. A.; Sagt, C. M. J.; Schmoll, M.; Sun, J. B.; Ussery, D.; Varga, J.; Vervecken, W.; de Vondervoort, P. J. J. V.; Wedler, H.; Wosten, H. A. B.; Zeng, A. P.; van Ooyen, A. J. J.; Visser, J.; Stam, H. Genome sequencing and analysis of the

versatile cell factory *Aspergillus niger* CBS 513.88. *Nat Biotechnol*. **2007,** *25*:221-231.

57. Fotso, J.; Leslie, J. F.; Smith, J. S. Production of beauvericin, moniliformin, fusaproliferin, and fumonisins B_1, B_2, and B_3 by fifteen ex-type strains of *Fusarium* species. *Appl Environ Microbiol*. **2002,** *68*:5195-5197.

58. Leslie, J. F.; Zeller, K. A.; Lamprecht, S. C.; Rheeder, J. P.; Marasas, W. F. O. Toxicity, pathogenicity, and genetic differentiation of five species of *Fusarium* from sorghum and millet. *Phytopathology*. **2005,** *95*:275-283.

59. Sharma, R.; Thakur, R. P.; Senthilvel, S.; Nayak, S.; Reddy, S. V.; Rao, V. P.; Varshney, R. K. Identification and characterization of toxigenic fusaria associated with sorghum grain mold complex in India. *Mycopathologia*. **2011,** *171*:223-230.

60. Wulff, E. G.; Sorensen, J. L.; Lubeck, M.; Nielsen, K. F.; Thrane, U.; Torp, J. *Fusarium* spp. associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity. *Environ Microbiol*. **2010,** *12*:649-657.

61. Chelkowski, J.; Lew, H. *Fusarium* species of *Liseola* section-occurence in cereals and ability to produce fumonisins. *Microbiologie Aliments Nutr*. **1992,** *10*:49-53.

62. Proctor, R. H.; Plattner, R. D.; Brown, D. W.; Seo, J. A.; Lee, Y. W. Discontinuous distribution of fumonisin biosynthetic genes in the *Gibberella fujikuroi* species complex. *Mycol Res*. **2004,** *108*:815-822.

63. Stepien, L.; Koczyk, G.; Waskiewicz, A. FUM cluster divergence in fumonisins-producing *Fusarium* species. *Fungal Biology*. **2011,** *115*:112-123.

64. Sydenham, E. W.; Shephard, G. S.; Stockenstrom, S.; Rheeder, J. P.; Marasas, W. F. O.; van der Merwe, M. J. Production of fumonisin B analogues and related compounds by *Fusarium globosum*, a newly described species from corn. *J Agric Food Chem*. **1997,** *45*:4004-4010.

65. Yang, Y.; Bouras, N.; Yang, J.; Howard, R. J.; Strelkov, S. E. Mycotoxin production by isolates of *Fusarium lactis* from greenhouse sweet pepper (*Capsicum annuum*). *Int J Food Microbiol*. **2011,** *151*:150-156.

66. Musser, S. M.; Plattner, R. D. Fumonisin composition in cultures of *Fusarium moniliforme*, *Fusarium proliferatum*, and *Fusarium nygamai*. *J Agric Food Chem*. **1997,** *45*:1169-1173.

67. Magnoli, C. E.; Saenz, M. A.; Chiacchiera, S. M.; Dalcero, A. M. Natural occurrence of *Fusarium* species and fumonisin production by toxigenic strains isolated from poultry feeds in Argentina. *Mycopathologia*. **1999,** *145*:35-41.

68. Sewram, V.; Mshicileli, N.; Shephard, G. S.; Vismer, H. F.; Rheeder, J. P.; Lee, Y. W.; Leslie, J. F.; Marasas, W. F. O. Production of fumonisin B and C analogues by several *Fusarium* species. *J Agric Food Chem*. **2005,** *53*:4861-4866.

69. Abbas, H. K.; Ocamb, C. M. First report of production of fumonisin B_1 by *Fusarium polyphialidicum* collected from seeds of *Pinus strobus*. *Plant Dis*. **1995,** *79*:642.

70. Moretti, A.; Ferracane, L.; Somma, S.; Ricci, V.; Mule, G.; Susca, A.; Ritieni, A.; Logrieco, A. F. Identification, mycotoxin risk and pathogenicity of *Fusarium* species associated with fig endosepsis in Apulia, Italy. *Food Addit Contam A*. **2010,** *27*:718-728.

71. Abbas, H. K.; Ocamb, C. M.; Xie, W. P.; Mirocha, C. J.; Shier, W. T. First report of fumonisin B_1, fumonisin B_2, and fumonisin B_3 production by *Fusarium oxysporum* var *redolens*. *Plant Dis*. **1995,** *79*:968.

72. Leslie, J. F.; Plattner, R. D.; Desjardins, A. E.; Klittich, C. J. R. Fumonisin B_1 production by strains from different mating populations of *Gibberella fujikuroi* (*Fusarium s*ection *Liseola*). *Phytopathology*. **1992,** *82*:341-345.

73. Baird, R.; Abbas, H. K.; Windham, G.; Williams, P.; Baird, S.; Ma, P.; Kelley, R.; Hawkins, L.; Scruggs, M. Identification of select fumonisin forming *Fusarium* species using PCR applications of the polyketide synthase gene and its relationship to fumonisin production in vitro. *Int J Mol Sci*. **2008,** *9*:554-570.

74. Klittich, C. J. R.; Leslie, J. F.; Nelson, P. E.; Marasas, W. F. O. *Fusarium thapsinum* (*Gibberella thapsina*): A new species in section *Liseola* from sorghum. *Mycologia*. **1997,** *89*:643-652.

75. Sydenham, E. W.; Shephard, G. S.; Thiel, P. G.; Marasas, W. F. O.; Stockenstrom, S. Fumonisin contamination of commercial corn-based human foodstuffs. *J Agric Food Chem*. **1991,** *39*:2014-2018.

76. Voss, K.; Smith, G.; Haschek, W. Fumonisins: Toxicokinetics, mechanism of action and toxicity. *Anim Feed Sci Technol*. **2007,** *137*:299-325.

77. Marasas, W. F. O.; Kellerman, T. S.; Gelderblom, W. C. A.; Coetzer, J. A. W.; Thiel, P. G.; Vanderlugt, J. J. Leukoencephalomalacia in a horse induced by fumonisin B_1 isolated from *Fusarium moniliforme*. *Onderstepoort J Vet Res*. **1988,** *55*:197-203.

78. Kellerman, T. S.; Marasas, W. F. O.; Thiel, P. G.; Gelderblom, W. C. A.; Cawood, M.; Coetzer, J. A. W. Leukoencephalomalacia in 2 horses induced by oral dosing of fumonisin B_1. *Onderstepoort J Vet Res*. **1990,** *57*:269-275.

79. Harrison, L. R.; Colvin, B. M.; Greene, J. T.; Newman, L. E.; Cole, Jr. J. R. Pulmonary edema and hydrothorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium moniliforme*. *J Vet Diagn Invest*. **1990,** *2*:217-221.

80. Stockmann-Juvala, H.; Savolainen, K. A review of the toxic effects and mechanisms of action of fumonisin B_1. *Hum Exp Toxicol*. **2008,** *27*:799-809.

81. Ueno, Y.; Iijima, K.; Wang, S. D.; Sugiura, Y.; Sekijima, M.; Tanaka, T.; Chen, C.; Yu, S. Z. Fumonisins as a possible contributory risk factor for primary liver cancer: A 3-year study of corn harvested in Haimen, China, by HPLC and ELISA. *Food Chem Toxicol*. **1997,** *35*:1143-1150.

82. Makaula, A. N.; Marasas, W. F.; venter, F. S.; Badenhorst, C. J.; Swanevelder, S. Oesophageal and other cancer patterns in four selected districts of the Transkei, Southern Africa: 1985-1990. *Afr J Health Sci*. **1996,** *3*:11-15.

83. Hendricks, K. Fumonisins and neural tube defects in South Texas. *Epidemiology*. **1999,** *10*:198-200.

84. Marasas, W. F. O.; Riley, R. T.; Hendricks, K. A.; Stevens, V. L.; Sadler, T. W.; Gelineau-van Waes, J.; Missmer, S. A.; Cabrera, J.; Torres, O.; Gelderblom, W. C. A.; Allegood, J.; Martinez, C.; Maddox, J.; Miller, J. D.; Starr, L.; Sullards, M. C.; Roman, A. V.; Voss, K. A.; Wang, E.; Merrill, A. H. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: A potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. *J Nutr*. **2004,** *134*:711-716.

85. Missmer, S. A.; Suarez, L.; Felkner, M.; Wang, E.; Merrill, A. H.; Rothman, K. J.; Hendricks, K. A. Exposure to fumonisins and the occurrence of neural tube defects along the Texas-Mexico border. *Environ Health Perspect*. **2006,** *114*:237-241.

86. Gelineau-van Waes, J.; Starr, L.; Maddox, J.; Aleman, F.; Voss, K. A.; Wilberding, J.; Riley, R. T. Maternal fumonisin exposure and risk for neural tube defects: mechanisms in an in vivo mouse model. *Birth Defects Res A*. **2005,** *73*:487-497.

87. Fincham, J. E.; Marasas, W. F. O.; Taljaard, J. J. F.; Kriek, N. P. J.; Badenhorst, C. J.; Gelderblom, W. C. A.; Seier, J. V.; Smuts, C. M.; Faber, M.; Weight, M. J.; Slazus, W.; Woodroof, C. W.; Vanwyk, M. J.; Kruger, M.; Thiel, P. G. Atherogenic effects in a nonhuman primate of *Fusarium moniliforme* cultures added to a carbohydrate diet. *Atherosclerosis*. **1992,** *94*:13-25.

88. Wang, E.; Norred, W. P.; Bacon, C. W.; Riley, R. T.; Merrill, A. H. Inhibition of sphingolipid biosynthesis by fumonisins - Implications for diseases associated with *Fusarium moniliforme*. *J Biol Chem*. **1991,** *266*:14486-14490.

89. Riley, R. T.; Enongene, E.; Voss, K. A.; Norred, W. P.; Meredith, F. I.; Sharma, R. P.; Spitsbergen, J.; Williams, D. E.; Carlson, D. B.; Merrill, A. H. Sphingolipid perturbations as mechanisms for fumonisin carcinogenesis. *Environ Health Perspect*. **2001,** *109*:301-308.

90. Riley, R. T.; Voss, K. A. Differential sensitivity of rat kidney and liver to fumonisin toxicity: Organ-specific differences in toxin accumulation and sphingoid base metabolism. *Toxicol Sci*. **2006,** *92*:335-345.

91. Soriano, J. M.; Gonzalez, L.; Catala, A. I. Mechanism of action of sphingolipids and their metabolites in the toxicity of fumonisin B_1. *Prog Lipid Res*. **2005,** *44*:345-356.

92. Dragan, Y. P.; Bidlack, W. R.; Cohen, S. M.; Goldsworthy, T. L.; Hard, G. C.; Howard, P. C.; Riley, R. T.; Voss, K. A. Implications of apoptosis for toxicity, carcinogenicity, and risk assessment: Fumonisin B_1 as an example. *Toxicol Sci*. **2001,** *61*:6-17.

93. Fernandez-Surumay, G.; Osweiler, G. D.; Yaeger, M. J.; Rottinghaus, G. E.; Hendrich, S.; Buckley, L. K.; Murphy, P. A. Fumonisin B-glucose reaction products are less toxic when fed to swine. *J Agric Food Chem*. **2005,** *53*:4264-4271.

94. Howard, P. C.; Couch, L. H.; Patton, R. E.; Eppley, R. M.; Doerge, D. R.; Churchwell, M. I.; Marques, M. M.; Okerberg, C. V. Comparison of the toxicity of several fumonisin derivatives in a 28-day feeding study with female B6C3F(1) mice. *Toxicol Appl Pharm*. **2002,** *185*:153-165.

95. Norred, W. P.; Riley, R. T.; Meredith, F. I.; Poling, S. M.; Plattner, R. D. Instability of N-acetylated fumonisin B_1 (FA_1) and the impact on inhibition of ceramide synthase in rat liver slices. *Food Chem Toxicol*. **2001,** *39*:1071-1078.

96. Merrill, A. H.; Wang, E.; Vales, T. R.; Smith, E. R.; Schroeder, J. J.; Menaldino, D. S.; Alexander, C.; Crane, H. M.; Xia, J.; Liotta, D. C.; Meredith, F. I.; Riley, R. T. Fumonisin toxicity and sphingolipid biosynthesis*;* Plenum Press: New York, 1996.

97. Gelderblom, W. C. A.; Cawood, M. E.; Snyman, S. D.; Vleggaar, R.; Marasas, W. F. O. Structure activity relationships of fumonisins in short term carcinogenesis and cytotoxicity assays. *Food Chem Toxicol*. **1993,** *31*:407-414.

98. Norred, W. P.; Plattner, R. D.; Dombrinkkurtzman, M. A.; Meredith, F. I.; Riley, R. T. Mycotoxin-induced elevation of free sphingoid bases in precision-cut rat liver slices: Specificity of the response and structure-activity relationships. *Toxicol Appl Pharm*. **1997,** *147*:63-70.

99. Gelderblom, W. C. A.; Jaskiewicz, K.; Marasas, W. F. O.; Thiel, P. G.; Horak, R. M.; Vleggaar, R.; Kriek, N. P. J. Fumonisins - Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl Environ Microbiol*. **1988,** *54*:1806-1811.

100. Sydenham, E. W.; Gelderblom, W. C. A.; Thiel, P. G.; Marasas, W. F. O. Evidence for the natural occurrence of fumonisin B_1, a mycotoxin produced by *Fusarium moniliforme*, in corn. *J Agric Food Chem*. **1990,** *38*:285-290.

101. Plattner, R. D.; Norred, W. P.; Bacon, C. W.; Voss, K. A.; Peterson, R.; Shackelford, D. D.; Weisleder, D. A method of detection of fumonisins in corn samples associated with field cases of equine leukoencephalomalacia. *Mycologia*. **1990,** *82*:698-702.

102. Plattner, R. D.; Shackelford, D. D. Biosynthesis of labeled fumonisins in liquid cultures of *Fusarium moniliforme*. *Mycopathologia*. **1992,** *117*:17-22.

103. Plattner, R. D.; Branham, B. E. Labeled fumonisins - Production and use of fumonisin B_1 containing stable isotopes. *J AOAC Int*. **1994,** *77*:525-532.

104. Arranz, I.; Baeyens, W. R. G.; Van der Weken, G.; De Saeger, S.; Van Peteghem, C. Review: HPLC determination of fumonisin mycotoxins. *Crit Rev Food Sci*. **2004,** *44*:195-203.

105. Shephard, G. S. Chromatographic determination of the fumonisin mycotoxins. *J Chromatogr A*. **1998,** *815*:31-39.

106. Shephard, G. S.; Thiel, P. G.; Stockenstrom, S.; Sydenham, E. W. Worldwide survey of fumonisin contamination of corn and corn-based products. *J AOAC Int*. **1996,** *79*:671-687.

107. Krska, R.; Welzig, E.; Boudra, H. Analysis of *Fusarium* toxins in feed. *Anim Feed Sci Technol*. **2007,** *137*:241-264.

108. Sforza, S.; Dall'Asta, C.; Marchelli, R. Recent advances in mycotoxin determination in food and feed by hyphenated chromatographic techniques/mass spectrometry. *Mass Spectrom Rev*. **2006,** *25*:54-76.

109. Shephard, G.; Berthiller, F.; Burdaspal, P.; Crews, C.; Jonker, M.; Krska, R.; MacDonald, S.; Malone, B.; Maragos, C.; Sabino, M.; Solfrizzo, M.; van Egmond, H.; Whitaker, T. Developments in mycotoxin analysis: an update for 2009-2010. *World Mycotoxin J*. **2011,** *4*:3-28.

110. Krska, R.; Schubert-Ullrich, P.; Molinelli, A.; Sulyok, M.; Macdonald, S.; Crews, C. Mycotoxin analysis: An update. *Food Addit Contam A*. **2008,** *25*:152-163.

111. Zöllner, P.; Mayer-Helm, B. Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionisation mass spectrometry. *J Chromatogr A*. **2006,** *1136*:123-169.

112. Silva, L.; Fernandez-Franzon, M.; Font, G.; Pena, A.; Silveira, I.; Lino, C.; Manes, J. Analysis of fumonisins in corn-based food by liquid chromatography with fluorescence and mass spectrometry detectors. *Food Chem*. **2009,** *112*:1031-1037.

113. Mogensen, J. M.; Larsen, T. O.; Nielsen, K. F. Widespread occurence of the mycotoxin fumonisin B_2 in wine. *J Agric Food Chem*. **2010,** *58*:4853-4857.

114. Logrieco, A.; Ferracane, R.; Visconti, A.; Ritieni, A. Natural occurrence of fumonisin B_2 in red wine from Italy. *Food Addit Contam A*. **2010,** *27*:1136-1141.

115. Visconti, A.; Boenke, A.; Solfrizzo, M.; Pascale, M.; Doko, M. B. European intercomparison study for the determination of the fumonisins content in two maize materials. *Food Addit Contam A*. **1996,** *13*:909-927.

116. Sydenham, E. W.; Shephard, G. S.; Thiel, P. G. Liquid chromatographic determination of fumonisin B_1, fumonisin B_2, and fumonisin B_3 in foods and feeds. *J AOAC Int*. **1992,** *75*:313-318.

117. Yazar, S.; Omurtag, G. Z. Fumonisins, trichothecenes and zearalenone in cereals. *Int J Mol Sci*. **2008,** *9*:2062-2090.

118. Morgavi, D. P.; Riley, R. T. An historical overview of field disease outbreaks known or suspected to be caused by consumption of feeds contaminated with *Fusarium* toxins. *Anim Feed Sci Technol*. **2007,** *137*:201-212.

119. Bottalico, A.; Logrieco, A.; Ritieni, A.; Moretti, A.; Randazzo, G.; Corda, P. Beauvericin and fumonisin B_1 in preharvest *Fusarium moniliforme* maize ear rot in Sardinia. *Food Addit Contam A*. **1995,** *12*:599-607.

120. Abbas, H. K.; Cartwright, R. D.; Shier, W. T.; Abouzied, M. M.; Bird, C. B.; Rice, L. G.; Ross, P. F.; Sciumbato, G. L.; Meredith, F. I. Natural occurrence of fumonisins in rice with *Fusarium* sheath rot disease. *Plant Dis*. **1998,** *82*:22-25.

121. Shetty, P. H.; Bhat, R. V. Natural occurrence of fumonisin B_1 and its co-occurrence with aflatoxin B_1 in Indian sorghum, maize, and poultry feeds. *J Agric Food Chem*. **1997,** *45*:2170-2173.

122. Martins, M. L.; Martins, H. M.; Bernardo, F. Fumonisins B_1 and B_2 in black tea and medicinal plants. *J Food Prot*. **2001,** *64*:1268-1270.

123. Scott, P. M.; Lawrence, G. A. Analysis of beer for fumonisins. *J Food Prot*. **1995,** *58*:1379-1382.

124. Liu, C. L.; Liu, F. M.; Xu, W. N.; Kofoet, A.; Humpf, H. U.; Jiang, S. R. Occurrence of fumonisins B_1 and B_2 in asparagus from Shandong province, P.R. China. *Food Addit Contam A*. **2005,** *22*:673-676.

125. Njobeh, P. B.; Dutton, M. F.; Koch, S. H.; Chuturgoon, A. A.; Stoev, S. D.; Mosonik, J. S. Simultaneous occurrence of mycotoxins in human food commodities from Cameroon. *Mycotox Res*. **2010,** *26*:47-57.

126. Bertuzzi, T.; Rastelli, S.; Mulazzi, A.; Donadini, G.; Pietri, A. Mycotoxin occurrence in beer produced in several European countries. *Food Control*. **2011,** *22*:2059-2064.

127. Soleimany, F.; Jinap, S.; Abas, F. Determination of mycotoxins in cereals by liquid chromatography tandem mass spectrometry. *Food Chem*. **2012,** *130*:1055-1060.

128. Karbancioglu-Guler, F.; Heperkan, D. Natural occurrence of fumonisin B_1 in dried figs as an unexpected hazard. *Food Chem Toxicol*. **2009,** *47*:289-292.

129. Gazzotti, T.; Lugoboni, B.; Zironi, E.; Barbarossa, A.; Serraino, A.; Pagliuca, G. Determination of fumonisin B_1 in bovine milk by LC-MS/MS. *Food Control*. **2009,** *20*:1171-1174.

130. Sacchi, C.; Gonzalez, H. H. L.; Broggi, L. E.; Pacin, A.; Resnik, S. L.; Cano, G.; Taglieri, D. Fungal contamination and mycotoxin natural occurrence in oats for race horses feeding in Argentina. *Anim Feed Sci Technol*. **2009,** *152*:330-335.

131. Aoyama, K.; Nakajima, M.; Tabata, S.; Ishikuro, E.; Tanaka, T.; Norizuki, H.; Itoh, Y.; Fujita, K.; Kai, S.; Tsutsumi, T.; Takahashi, M.; Tanaka, H.; Iizuka, S.; Ogiso, M.; Maeda, M.; Yamaguchi, S.; Sugiyama, K. i.; Sugita-Konishi, Y.; Kumagai, S. Four-year surveillance for ochratoxin A and fumonisins in retail foods in Japan. *J Food Prot*. **2010,** *73*:344-352.

132. Bansal, J.; Pantazopoulos, P.; Tam, J.; Cavlovic, P.; Kwong, K.; Turcotte, A.; Lau, B.; Scott, P. Surveys of rice sold in Canada for aflatoxins, ochratoxin A and fumonisins. *Food Addit Contam A*. **2011,** *28*:767-774.

133. Roger, D. D. Deoxynivanol (DON) and fumonisins B_1 (FB_1) in artisanal sorghum opaque beer brewed in north Cameroon. *Afr J Microbiol Res*. **2011,** *5*:1565-1567.

134. Ghali, R.; Ghorbel, H.; Hedilli, A. Fumonisin determination in Tunisian foods and feeds. ELISA and HPLC methods comparison. *J Agric Food Chem*. **2009,** *57*:3955-3960.

135. Monbaliu, S.; Wu, A. B.; Zhang, D. B.; Van Peteghem, C.; De Saeger, S. Multimycotoxin UPLC-MS/MS for tea, herbal Infusions and the derived drinkable products. *J Agric Food Chem*. **2010,** *58*:12664-12671.

136. Varga, J.; Kocsube, S.; Suri, K.; Szigeti, G.; Szekeres, A.; Varga, M.; Toth, B.; Bartok, T. Fumonisin contamination and fumonisin producing black Aspergilli in dried vine fruits of different origin. *Int J Food Microbiol*. **2010,** *143*:143-149.

137. Logrieco, A.; Ferracane, R.; Haidukowsky, M.; Cozzi, G.; Visconti, A.; Ritieni, A. Fumonisin B_2 production by *Aspergillus niger* from grapes and natural occurrence in must. *Food Addit Contam A*. **2009,** *26*:1495-1500.

138. Geiser, D. M.; Klich, M. A.; Frisvad, J. C.; Peterson, S. W.; Varga, J.; Samson, R. A. The current status of species recognition and identification in *Aspergillus*. *Stud Mycol*. **2007,** *59*:1-10.

139. Samson, R. A.; Houbraken, J. A. M. P.; Kuijpers, A. F. A.; Frank, J. M.; Frisvad, J. C. New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. *Stud Mycol*. **2004,** *50*:45-61.

140. Abarca, M. L.; Accensi, F.; Cano, J.; Cabañes, F. J. Taxonomy and significance of black aspergilli. *Anton Leeuw Int J G*. **2004,** *86*:33-49.

141. Kozakiewicz, Z. *Aspergillus* species on stored products. *Mycol Papers*. **1989,** *161*:1-188.

142. Ferracin, L. M.; Frisvad, J. C.; Taniwaki, M. H.; Iamanaka, B. T.; Sartori, D.; Schapovaloff, M. E.; Pelegrinelli Fungaro, M. H. Genetic relationships among strains of the *Aspergillus niger* aggregate. *Braz Arch Biol Technol*. **2009,** *52*:241-248.

143. Pitt, J. I.; Hocking, A. D. Fungi and food spoilage; 2nd ed.; Blackie Academic and Professional: London, U.K., 1997.

144. Samson, R. A.; Noonim, P.; Meijer, M.; Houbraken, J.; Frisvad, J. C.; Varga, J. Diagnostic tools to identify black aspergilli. *Stud Mycol*. **2007,** *59*:129-145.

145. Varga, J.; Frisvad, J.; Kocsube, S.; Brankovics, B.; Toth, B.; Szigeti, G.; Samson, R. New and revisited species in *Aspergillus* section *Nigri*. *Stud Mycol*. **2011,**1-17.

146. Frisvad, J. C.; Larsen, T. O.; Thrane, U.; Meijer, M.; Varga, J.; Samson, R. A.; Nielsen, K. F. Fumonisin and ochratoxin production in industrial *Aspergillus niger s*trains. *Plos One*. **2011,** *6*:e23496.

147. Samson, R. A.; Houbraken, J.; Thrane, U.; Frisvad, J. C.; Andersen, B. Food and indoor fungi; CBS-KNAW Fungal Biodiversity Centre: Uthrecht, The Netherlands, 2010.

148. Kusters-Van, S. O. M. E.; Samson, R. A.; Visser, J. The use of RFLP analysis in classification of the black aspergilli reinterpretation of the *Aspergillus niger* aggregate. *Curr Genet*. **1991,** *19*:21-26.

149. Al-Musallam, A. Revision of the black *Aspergillus* species; Thesis ed.; Rijksuniversiteit: Utrecht, 1980.

150. Silva, D. M.; Batista, L. R.; Rezende, E. F.; Fungaro, M. H.; Sartori, D.; Alves, E. Identification of fungi of the genus *Aspergillus* section *Nigri* using polyphasic taxonomy. *Braz J Microbiol*. **2011,** *42*:761-773.

151. de Vries, R. P.; Visser, J. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol Mol Biol Rev*. **2001,** *65*:497-522.

152. Schuster, E.; Dunn-Coleman, N.; Frisvad, J. C.; van Dijck, P. W. M. On the safety of *Aspergillus niger* - a review. *Appl Microbiol Biotech*. **2002,** *59*:426-435.

153. Ward, O. P.; Qin, W. M.; Dhanjoon, J.; Ye, J.; Singh, A. Physiology and biotechnology of *Aspergillus*. *Adv Appl Microbiol*. **2006,** *58*:1-75.

154. Blumenthal, C. Z. Production of toxic metabolites in *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. *Regul Toxicol Pharmacol*. **2004,** *39*:214-228.

155. Dhillon, G. S.; Brar, S. K.; Verma, M.; Tyagi, R. D. Recent advances in citric acid bio-production and recovery. *Food Bioprocess Tech*. **2011,** *4*:505-529.

156. Biesebekei, R. T.; Record, E. Scientific advances with *Aspergillus* species that are used for food and biotech applications. *Microbes Environ*. **2008,** *23*:177-181.

157. Punt, P. J.; van Biezen, N.; Conesa, A.; Albers, A.; Mangnus, J.; van den Hondel, C. Filamentous fungi as cell factories for heterologous protein production. *Trends Biotechnol*. **2002,** *20*:200-206.

158. Nielsen, K. F.; Mogensen, J. M.; Johansen, M.; Larsen, T. O.; Frisvad, J. C. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Anal Bioanal Chem*. **2009,** *395*:1225-1242.

159. de Vries, R. P.; Frisvad, J. C.; van de Vondervoort, P. J. I.; Burgers, K.; Kuijpers, A. F. A.; Samson, R. A.; Visser, J. *Aspergillus vadensis*, a new species of the group of black Aspergilli. *Anton Leeuw Int J G*. **2005,** *87*:195-203.

160. Battilani, P.; Pietri, A.; Bertuzzi, T.; Languasco, L.; Giorni, P.; Kozakiewicz, Z. Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. *J Food Prot*. **2003,** *66*:633-636.

161. Dalcero, A.; Magnoli, C.; Hallak, C.; Chiacchiera, S. M.; Palacio, G.; Rosa, C. A. R. Detection of ochratoxin A in animal feeds and capacity to produce this mycotoxin by *Aspergillus* section *Nigri* in Argentina. *Food Addit Contam A*. **2002,** *19*:1065-1072.

162. Medina, A.; Mateo, R.; Lopez-Ocana, L.; Valle-Algarra, F. M.; Jimenez, M. Study of Spanish grape mycobiota and ochratoxin a production by isolates of *Aspergillus tubingensis* and other members of *Aspergillus* section *Nigri*. *Appl Environ Microbiol*. **2005,** *71*:4696-4702.

163. Abarca, M. L.; Bragulat, M. R.; Castella, G.; Cabañes, F. J. Ochratoxin A Production by strains of *Aspergillus niger* var. *niger*. *Appl Environ Microbiol*. **1994,** *60*:2650-2652.

164. Abarca, M. L.; Accensi, F.; Bragulat, M. R.; Cabanes, F. J. Current importance of ochratoxin A-producing *Aspergillus* spp. *J Food Prot*. **2001,** *64*:903-906.

165. Palumbo, J. D.; O'Keeffe, T. L.; McGarvey, J. A. Incidence of fumonisin B_2 production within *Aspergillus* Section *Nigri* populations isolated from California raisins. *J Food Prot*. **2011,** *74*:672-675.

166. Frisvad, J. C.; Samson, R. A. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium* - A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Stud Mycol*. **2004,** *49*:1-173.

167. Bullerman, L. B. Screening medium and method to detect several mycotoxins in mold cultures. *J Milk Food Technol*. **1974,** *37*:1-3.

168. Esteban, A.; Abarca, M. L.; Bragulat, M. R.; Cabañes, F. J. Effects of temperature and incubation time on production of ochratoxin A by black aspergilli. *Res Microbiol*. **2004,** *155*:861-866.

169. Alborch, L.; Bragulat, M.; Abarca, M.; Cabanes, F. Effect of water activity, temperature and incubation time on growth and ochratoxin A production by *Aspergillus niger* and *Aspergillus carbonarius* on maize kernels. *Int J Food Microbiol*. **2011,** *147*:53-57.

170. Leong, S. L. L.; Hocking, A. D.; Scott, E. S. Effect of temperature and water activity on growth and ochratoxin A production by Australian *Aspergillus carbonarius* and *A. niger* isolates on a simulated grape juice medium. *Int J Food Microbiol*. **2006,** *110*:209-216.

171. Esteban, A.; Abarca, M. L.; Bragulat, M. R.; Cabañes, F. J. Effect of water activity on ochratoxin A production by *Aspergillus niger* aggregate species. *Int J Food Microbiol*. **2006,** *108*:188-195.

172. Marin, S.; Sanchis, V.; Magan, N. Water activity, temperature, and pH effects on growth of *Fusarium moniliforme* and *Fusarium proliferatum* isolates from maize. *Can J Microbiol*. **1995,** *41*:1063-1070.

173. Etcheverry, M.; Torres, A.; Ramirez, M. L.; Chulze, S.; Magan, N. In vitro control of growth and fumonisin production by *Fusarium verticillioides* and *F. proliferatum* using antioxidants under different water availability and temperature regimes. *J Appl Microbiol*. **2002,** *92*:624-632.

174. Cabañes, F. J.; Accensi, F.; Bragulat, M. R.; Abarca, M. L.; Castella, G.; Minguez, S.; Pons, A. What is the source of ochratoxin A in wine? *Int J Food Microbiol*. **2002,** *79*:213-215.

175. Battilani, P.; Pietri, A. Ochratoxin A in grapes and wine. *Eur J Plant Pathol*. **2002,** *108*:639-643.

176. Sage, L.; Krivobok, S.; Delbos, E.; Seigle-Murandi, F.; Creppy, E. E. Fungal flora and ochratoxin A production in grapes and musts from France. *J Agric Food Chem*. **2002,** *50*:1306-1311.

177. Abrunhosa, L.; Calado, T.; Venancio, A. Incidence of fumonisin $B_{2\,p}$roduction by *Aspergillus niger* in portuguese wine regions. *J Agric Food Chem*. **2011,** *59*:7514-7518.

178. Zimmerli, B.; Dick, R. Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. *Food Addit Contam A*. **1996,** *13*:655-668.

179. Abarca, M. L.; Accensi, F.; Bragulat, M. R.; Castella, G.; Cabañes, F. J. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *J Food Prot*. **2003,** *66*:504-506.

180. Magnoli, C.; Astoreca, A.; Ponsone, L.; Combina, M.; Palacio, G.; Rosa, C. A. R.; Dalcero, A. M. Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. *Lett Appl Microbiol*. **2004,** *39*:326-331.

181. Otteneder, H.; Majerus, P. Occurrence of ochratoxin A (OTA) in wines: influence of the type of wine and its geographical origin. *Food Addit Contam A*. **2000,** *17*:793-798.

182. Burdaspal, P.; Legarda, T. Ochratoxin A in wines and grape products originated from Spain and other european countries. *Alimentaria*. **1999,** *36*:107-113.

183. Majerus, P.; Otteneder, H. Detection and occurrence of ochratoxin A in wine and grapejuice. *Deut Lebensm-Rundsch*. **1996,** *92*:388-390.

184. Esmaiili, M.; Sotudeh-Gharebagh, R.; Cronin, K.; Mousavi, M. A.; Rezazadeh, G. Grape drying: A review. *Food Rev Int*. **2007,** *23*:257-280.

185. Leong, S. L.; Hocking, A. D.; Pitt, J. I. Occurrence of fruit rot fungi (Aspergillus section Nigri) on some drying varieties of irrigated grapes. *Australian Journal of Grape and Wine Research*. **2004,** *10*:83-88.

186. Sano, M.; Takenaka, Y.; Kojima, R.; Saito, S.; Tomita, I.; Katou, M.; Shibuya, S. Effects of pu-erh tea on lipid metabolism in rats. *Chem Pharm Bull*. **1986,** *34*:221-228.

187. Abe, M.; Takaoka, N.; Idemoto, Y.; Takagi, C.; Imai, T.; Nakasaki, K. Characteristic fungi observed in the fermentation process for Puer tea. *Int J Food Microbiol*. **2008,** *124*:199-203.

188. Elshafie, A. E.; Al Lawatia, T.; Al Bahry, S. Fungi associated with black tea and tea quality in the sultanate of Oman. *Mycopathologia*. **1999,** *145*:89-93.

189. Mo, H. Z.; Xu, X. Q.; Yan, M. C.; Zhu, Y. Microbiological analysis and antibacterial effects of the indigenous fermented Puer tea. *Agro Food Ind Hi Tec*. **2005,** *16*:16-18.

190. Xu, X.; Yan, M.; Zhu, Y. Influence of fungal fermentation on the development of volatile compounds in the Puer tea manufacturing process. *Eng Life Sci*. **2005,** *5*:382-386.

191. Wang, D.; Xu, K.; Zhong, Y.; Luo, X.; Xiao, R.; Hou, Y.; Bao, W.; Yang, W.; Yan, H.; Yao, P.; Liu, L. Acute and subchronic oral toxicities of Pu-erh black tea extract in Sprague-Dawley rats. *Journal of Ethnopharmacology*. **2011,** *134*:156-164.

192. Mphande, F. A.; Siame, B. A.; Taylor, J. E. Fungi, aflatoxins, and cyclopiazonic acid associated with peanut retailing in Botswana. *J Food Prot*. **2004,** *67*:96-102.

193. Youssef, M.; El-Maghraby, O.; Ibrahim, Y. Mycobiota and mycotoxins of egyptian peanut (*Arachis hypogeae* L.) seeds. *Int J Bot*. **2008,** *4*:ISSN.

194. Jimenez, M.; Mateo, R.; Querol, A.; Huerta, T.; Hernandez, E. Mycotoxins and mycotoxigenic molds in nuts and sunflower seeds for human consumption. *Mycopathologia*. **1991,** *115*:121-127.

195. Ismail, M. A. Deterioration and spoilage of peanuts and desiccated coconuts from two sub-Saharan tropical East African countries due to the associated mycobiota and their degradative enzymes. *Mycopathologia*. **2000,** *150*:67-84.

196. Pitt, J. I.; Hocking, A. D.; Bhudhasamai, K.; Miscamble, B. F.; Wheeler, K. A.; Tanboonek, P. The normal mycoflora of commodities from Thailand . 1. Nuts and oilseeds. *Int J Food Microbiol*. **1993,** *20*:211-226.

197. Rheeder, J. P.; Marasas, W. F. O.; Thiel, P. G.; Sydenham, E. W.; Shephard, G. S.; Vanschalkwyk, D. J. *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. *Phytopathology*. **1992,** *82*:353-357.

198. van der Westhuizen, L.; Shephard, G. S.; Rheeder, J. P.; Burger, H. M.; Gelderblom, W. C. A.; Wild, C. P.; Gong, Y. Y. Optimising sorting and washing of home-grown maize to reduce fumonisin contamination under laboratory-controlled conditions. *Food Control*. **2011,** *22*:396-400.

199. Vishwanath, V.; Sulyok, M.; Labuda, R.; Bicker, W.; Krska, R. Simultaneous determination of 186 fungal and bacterial metabolites in indoor matrices by liquid chromatography/tandem mass spectrometry. *Anal Bioanal Chem*. **2009,** *395*:1355-1372.

200. Sala, N.; Sanchis, V.; Vilaro, P.; Viladrich, R.; Torres, M.; Vinas, I.; Canela, R. Fumonisin producing capacity of *Fusarium* strains isolated from cereals in Spain. *J Food Prot*. **1994,** *57*:915-917.

201. Bacon, C. W.; Porter, J. K.; Norred, W. P.; Leslie, J. F. Production of fusaric acid by *Fusarium* species. *Appl Environ Microbiol*. **1996,** *62*:4039-4043.

202. Diniz, S. P. S. S.; Oliveira, R. C. Effects of fusaric acid on Zea mays L. seedlings. *Phyton-Int J Exp Bot*. **2009,** *78*:155-160.

203. Antilla, L.; Cotty, P. J. The ARS-ACRPC partnership to control aflatoxin in Arizona: Current status. *Mycopathologia*. **2002,** *155*:64.

204. Cleveland, T. E.; Dowd, P. F.; Desjardins, A. E.; Bhatnagar, D.; Cotty, P. J. United States Department of Agriculture - Agricultural Research Service research on pre-harvest prevention of mycotoxins and mycotoxigenic fungi in US crops. *Pest Manag Sci*. **2003,** *59*:629-642.

205. Abbas, H. K.; Zablotowicz, R. M.; Bruns, H. A.; Abel, C. A. Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillus flavus* isolates. *Biocontrol Sci Techn*. **2006,** *16*:437-449.

206. Burmeister, H. R.; Bennett, G. A.; Vesonder, R. F.; Hesselti, C. W. Antibiotic produced by *Fusarium equiseti* NRRL-5537. *Antimicrob Agents Ch*. **1974,** *5*:634-639.

207. Shephard, G. S. Risk assessment of aflatoxins in food in Africa. *Food Addit Contam A*. **2008,** *25*:1246-1256.

208. van der Westhuizen, L.; Shephard, G. S.; Burger, H. M.; Rheeder, J. P.; Gelderblom, W. C. A.; Wild, C. P.; Gong, Y. Y. Fumonisin B_1 as a urinary biomarker of exposure in a maize

intervention study among South African subsistence farmers. *Cancer Epidem Biomar*. **2011,** *20*:483-489.

209. van der Westhuizen, L.; Shephard, G. S.; Rheeder, J. P.; Burger, H. M.; Gelderblom, W. C. A.; Wild, C. P.; Gong, Y. Y. Simple intervention method to reduce fumonisin exposure in a subsistence maize-farming community in South Africa. *Food Addit Contam A*. **2010,** *27*:1582-1588.

210. Survase, S. A.; Kagliwal, L. D.; Annapure, U. S.; Singhal, R. S. Cyclosporin A - A review on fermentative production, downstream processing and pharmacological applications. *Biotechnol Adv*. **2011,** *29*:418-435.

211. Frisvad, J. C. A selective and indicative medium for groups of *Penicillium viridicatum* producing different mycotoxins in cereals. *J Appl Bacteriol*. **1983,** *54*:409-416.

212. Simmons, E. G. *Alternaria*. An identification manual*;* CBS Fungal Biodiversity Centre: Utrecht, The Netherlands, 2007.

213. Hocking, A. D.; Pitt, J. I. Dichloran glycerol medium for enumeration of xerophilic fungi from low moisture foods. *Appl Environ Microbiol*. **1980,** *39*:488-492.

214. Strasser, H.; Vey, A.; Butt, T. M. Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium, Tolypocladium* and *Beauveria* species? *Biocontrol Sci Techn*. **2000,** *10*:717-735.

215. Butchko, R. A.; Plattner, R. D.; Proctor, R. H. Deletion analysis of FUM genes involved in tricarballylic ester formation during fumonisin biosynthesis. *J Agric Food Chem*. **2006,** *54*:9398-9404.

216. Liao, W.; McNutt, M. A.; Zhu, W. G. The comet assay: A sensitive method for detecting DNA damage in individual cells. *Methods*. **2009,** *48*:46-53.

217. Klaric, M. S.; Pepeljnjak, S.; Rozgaj, R. Genotoxicity of fumonisin B_1, beauvericin and ochratoxin A in porcine kidney PK15 cells: Effects of individual and combined treatment. *Croat Chem Acta*. **2008,** *81*:139-146.

218. Knudsen, P. B.; Mogensen, J. M.; Larsen, T. O.; Nielsen, K. F. Occurrence of fumonisins B_2 and B_4 in retail raisins. *J Agric Food Chem*. **2010,** *59*:772-776.

219. Nielsen, K. F.; Logrieco, A. F. Letter to the editor on 'Fumonisin contamination and fumonisin producing black Aspergilli in dried vine fruits of different origin, IJFM. 143:143-149'. *Int J Food Microbiol*. **2012,** *152*:45.

220. Nielsen, K. F.; Logrieco, A. F. Rebuttal to the response letter to our letter to the editor on 'Fumonisin contamination and fumonisin producing black Aspergilli in dried vine fruits of different origin published in International Journal of Food Microbiology, 143:143-149'. *Int J Food Microbiol*. **2012,** *152*:49-50.

221. Visconti, A.; Haidukowski, E. M.; Pascale, M.; Silvestri, M. Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. *Toxicol Lett*. **2004,** *153*:181-189.

222. Food and Agricultural Organization Code of practice for the prevention and reduction of mycotoxin contamination in cerals, including annexes on ochratoxin A, zearalenone, fumonisins and tricothecenes. In *Codex Alimentarius - Cereals, Pulses, Legumes and Vegetable proteins.* 1st edition. World Health Organization. Rome. 2007; pp 68-77.

223. Ghori, M. I.; Ahmed, S.; Malana, M. A.; Jamil, A. Corn stover-enhanced cellulase production by *Aspergillus niger* NRRL 567. *Afr J Biotechnol*. **2011,** *10*:5878-5886.

224. Adham, N. Z.; Ahmed, E. Extracellular lipase of *Aspergillus niger* NRRL3; production, partial purification and properties. *Indian J Microbiol*. **2009,** *49*:77-83.

225. Barrington, S.; Kim, J. S.; Wang, L.; Kim, J. W. Optimization of citric acid production by *Aspergillus niger* NRRL 567 grown in a column bioreactor. *Korean J Chem Eng*. **2009,** *26*:422-427.

226. Guilherme, A.; Pinto, G.; Rodrigues, S. Optimization of trace metals concentration on citric acid production by *Aspergillus niger* NRRL 2001. *Food Bioprocess Tech*. **2008,** *1*:246-253.

227. Oboh, G.; Akindahunsi, A. A.; Oshodi, A. A. Nutrient and anti-nutrient contents of *Aspergillus niger* fermented cassava products (flour and gari). *J Food Compos Anal*. **2002,** *15*:617-622.

228. Singh, A.; Abidi, A. B.; Agrawal, A. K.; Darmwal, N. S. Single cell protein production by *Aspergillus niger* and its evaluation. *Zbl Mikrobiol*. **1991,** *146*:181-184.

229. Aranda, M.; Perez-Alzola, L.; Ellahuene, M.; Sepulveda, C. Assessment of in vitro mutagenicity in Salmonella and in vivo genotoxicity in mice of the mycotoxin fumonisin B-1. *Mutagenesis*. **2000,** *15*:469-471.

230. Knasmuller, S.; Bresgen, N.; Kassie, F.; MerschSundermann, V.; Gelderblom, W.; Zohrer, E.; Eckl, P. M. Genotoxic effects of three Fusarium mycotoxins, fumonisin B-1, moniliformin and vomitoxin in bacteria and in primary cultures of rat hepatocytes. *Mutat Res-Gen Tox En*. **1997,** *391*:39-48.

231. Waldeck, B. Three-dimensional pharmacology, a subject ranging from ignorance to overstatements. *Pharmacol Toxicol*. **2003,** *93*:203-210.

6 Original manuscripts (1-9)

Paper 1: **J.M. Mogensen**, K.F. Nielsen, R.A. Samson, J.C. Frisvad and U. Thrane. Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* spp. BMC Microbiology. 2009. 9:281

Paper 2: **J.M. Mogensen,** J.C. Frisvad, U. Thrane, K.F. Nielsen. Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. Journal of Agricultural and Food Chemistry. 2010, 58:954-958

Paper 3: **J.M. Mogensen**, T.O. Larsen, K.F. Nielsen. Widespread occurrence of the mycotoxin Fumonisin B_2 in wine. Journal of Agricultural and Food Chemistry. 2010, 58:4583-4587

Paper 4: P. B. Knudsen, **J. M. Mogensen**, T. O. Larsen and K. F. Nielsen. Occurrence of fumonisins B_2 and B_4 in retail raisins. Journal of Agricultural and Food Chemistry. 2011, 59:772-776

Paper 5: **J.M. Mogensen**. J. Varga, U. Thrane and J.C. Frisvad. *Aspergillus acidus* from Puerh tea and black tea does not produce ochratoxin A and fumonisin B_2. International Journal of Food Microbiology. 2009. 132:141-144.

Paper 6: **J. M. Mogensen**, E. O. Søndergaard and K. F. Nielsen. Studies on fumonisins in peanuts and peanut butter. Journal of Agricultural and Food Chemistry. Submitted.

Paper 7: **J. M. Mogensen**, S. M. Sørensen, M. Sulyok, L. van der Westhuizen, G. Shephard, J.C. Frisvad, U. Thrane, R. Krska, K. F. Nielsen. Single kernel analysis of fumonisins and other fungal metabolites in maize from South African subsistence farmers. Food Additives & Contaminants. 2011. 28:1724-1734.

Paper 8: **J. M. Mogensen**, K. A. Møller, P. von Freiesleben, R. Labuda, E. Varga, M. Sulyok, A. Kubátová, U. Thrane, B. Andersen, K. F. Nielsen. Production of Fumonisins B_2 and B_4 in *Tolypocladium* species. Journal of Industrial Microbiology and Biotechnology. 2011, 38:1329-1335.

Paper 9: K.F. Nielsen, **J.M. Mogensen**, M. Johansen, T.O. Larsen and J.C. Frisvad. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Analytical & Bioanalytical Chemistry. 2009. 395:1225-1242.

Paper 1

” Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* species”

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Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* species

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Abstract

Background: Fumonisins are economically important mycotoxins which until recently were considered to originate from only a few *Fusarium* species. However recently a putative fumonisin gene cluster was discovered in two different *Aspergillus niger* strains followed by detection of an actual fumonisin B_2 (FB_2) production in four strains of this biotechnologically important workhorse.

Results: In the present study, a screening of 5 *A. niger* strains and 25 assumed fumonisin producing *Fusarium* strains from 6 species, showed that all 5 *A. niger* strains produced FB_2 and 23 of 25 *Fusarium* produced fumonisin B_1 and other isoforms (fumonisin B_2 and B_3). Five *A. niger* and five *Fusarium* spp. were incubated at six different temperatures from 15-42°C on Czapek Yeast Agar +5% salt or Potato Dextrose Agar. *A. niger* had the highest production of FB_2 at 25-30°C whereas *Fusarium* spp. had the maximal production of FB_1 and FB_2 at 20-25°C. Addition of 2.5-5% NaCl, or 10-20% sucrose increased the FB_2 production of *A. niger*, whereas addition of glycerol reduced FB_2 production. All three water activity lowering solutes reduced the fumonisin production of the *Fusarium* species.

Conclusion: The present study shows that the regulation of fumonisin production is very different in *A. niger* and *Fusarium*, and that food and feeds preserved by addition of sugar or salts may be good substrates for fumonisin B_2 production by *A. niger*.

Background

The fumonisins were discovered in 1988 and are divided in four series A, B, C, P [1-3]with the B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) as the most abundant naturally occurring homologues [4,5]. They were first isolated from *Fusarium verticillioides* (= *F. moniliforme* pro parte [6]) strain MRC 826 by Gelderblom *et al*. [7]. FB_1 is mainly produced by *F. verticillioides* and *F. proliferatum* [8]. However, production of type B fumonisins by other *Fusarium* spp. has also been reported, e.g. from *F. dlaminii*, *F. napiforme*, *F. nygamai* and *F. oxysporum* [8-10]. Fumonisins are important mycotoxins because they are suspected to cause human and animal toxicoses by the consumption of contaminated corn-based food and feeds [11]. Fumonisins have been shown to induce outbreaks of equine leukoencephalomalacia in horses and pulmonary edema and hydrothorax in pigs

[5,12]. The fumonisins are structurally similar to sphingolipids and have shown to inhibit the sphingolipid biosynthesis via the ceramide synthase pathway [13,14]. To avoid possible health risks, the U.S. Food and Drug Administration recommends that corn products should not be used for human consumption when contaminated with more than 2-4 mg/kg total fumonisins (depending on the product) [15]. whereas EEC has a regulatory limit of 0.2-2 mg/kg (depending on the product) [16].

Fumonisins produced by *Fusarium* species have been isolated from corn [1] and corn based products [11] such as tortillas [17] and beer [18], as well as other commodities like rice [19], black tea leaves [20], asparagus [21] and pine nuts [22].

Factors that affect the production of fumonisins in *Fusarium* have been well studied, and include solid substrates [23], liquid substrates [24], temperature [25-27], water activity (a_w) [27,28], pH [29], addition of nitrogen repressor [30], aeration of the substrate [29] and addition of fumonisin precursors [31], but often corn kernels have been used as substrate since corn is the primary crop infected with *F. verticillioides*.

Recently putative homologues to the *F. verticillioides* fumonisin gene cluster were found in two different *Aspergillus niger* genomes [32,33], and it was subsequently shown that three full genome sequenced strains and the ex type strain of *A. niger* actually can produce FB_2 in comparable amounts to *Fusarium* strains [34] when grown on agar substrates with high amounts of sugar, glycerol or NaCl.

This was followed by the discovery of additional FB_4 production (~20% the amount of FB_2) by *A. niger* [35] in agar cultures and naturally *A. niger* contaminated Thai coffee beans [35].

The objectives of the present work were to *i*) screen *A. niger* and *Fusarium* strains, for production of FB_1, FB_2 and FB_3 on three different agar substrates, *ii*) investigate the effect of incubation temperature on the production of fumonisins and *iii*) study the effect of the solutes glycerol, NaCl and sucrose on the production of fumonisins. The current work is performed on agar media instead of natural substrates in order to more easily asses the water activity.

Results

Optimization of extraction

The efficiency of five different extraction solvents to extract FB_2 from *A. niger* (NRRL 567) varied significantly, with methanol:water (3:1) being most efficient, followed by acetonitrile:water (3:1) with a 20% lower efficiency and methanol:dichloromethane:ethyl acetate (1:2:3) (30% lower efficiency). The use of water (25°C) and hot water (100°C) was not suitable for extraction of FB_2 from *A. niger* NRRL 567 with a relative efficiency of <1%, when compared to methanol:water. The most effective solvents concerning *F. verticillioides* IBT 9400 were acetonitrile:water (3:1) as the best followed by methanol:water (3:1)(98%) and water at room temperature with an efficiency of 93%. The use of hot water as extraction solvent was less efficient (76%), compared to methanol:water. The acidic methanol:dichloromethane:ethyl acetate (1:2:3), had the lowest extraction efficiency with 26%. The difference between each of the two replicates for all incidences was in the interval 1-40% with an average of 10%.

Validation of methanol-water extraction from A. niger

The recovery of FB_2 from two spiked non-FB_2 producing strains showed a recovery of 75% ± 10% (IBT 20381) and 85% ± 10% (IBT 19345). The calibration curves from standards and spiked samples, used to calculate recovery from all had R^2 better than 0.995. The relative standard deviation (RSD) of the extracted amounts of FB_2 from the 8 isolates (n = 5) varied within 4-50%, with an average RSD of 20%. LOD were found to be 0.1 μg/cm^2 fungal culture.

Screening of strains for fumonisin production

The results from the screening experiment are shown in table 1. The *A. niger* strains were able to produce FB_2 on all three substrates, with the highest production on RC and CYAS. None of the *A. niger* strains produced detectable amounts of neither FB_1 nor FB_3. LC-MS/MS analyses have shown that FB_2 is produced along with FB_4, although the amount of FB_4 normally lies in the range 5-20% of the FB_2 amounts [35].

Of the *A. niger* strains, NRRL 567 had the highest production of FB_2 on RC and CYAS. But on PDA the three strains, NRRL 567, NRRL 2001 and IBT 24631, produced very similar amounts of fumonisins, although lower than RC and CYAS. The FB_2 production on RC and CYAS of *A. niger* NRRL 2001, IBT 24631 and 24634 differed only slightly, whereas FB_2 production by the other two, NRRL 567 and NRRL 3 were clearly favored by growth on RC. All *Fusarium* spp. with the exception of the two *F. dlaminii* strains IBT 2937 and IBT 2938 produced fumonisins under these conditions. Six *Fusarium* strains, *F. napiforme* IBT 2932, *F. proliferatum* IBT 9109 and IBT 9337, *F. verticillioides* IBT 9496 and *F. nygamai* IBT 9395 produced amounts close to the detection limit of FB_1 on RC; in addition IBT 2932 also produced fumonisins on PDA. *F. nygamai* IBT 2934, IBT 8554 and IBT 8557 showed a higher production of FB_2 than FB_1 on PDA (data not shown), and *F. proliferatum* IBT 9397 had the highest concentration of total fumonisin

Table 1: Fumonisin production by *Aspergillus niger* and *Fusarium* spp. on CYAS, PDA and RC after 7 days growth at 25°C.

Fungi	Isolate	CYAS g/ml	RC g/ml	PDA g/ml
A. niger	NRRL 3	2.9 ± 0.4	7.9 ± 0.7	0.86 ± 0.02
	NRRL 567	25 ± 0.9	36 ± 2	1.9 ± 0.5
	NRRL 2001	7.6 ± 0.7	6.1 ± 0.9	3.1 ± 0.3
	IBT 24631	5.2 ± 0.2	6.7 ± 0.7	1.3 ± 0.6
	IBT 24634	6.4 ± 0.1	5.3 ± 0.4	0.46 ± 0.02
F. proliferatum	IBT 8904	n.d.	9.9 ± 3	21 ± 0.5
	IBT 9109	n.d.	0.028 ± 0.007	n.d.
	IBT 9337	n.d.	0.021 ± 0.01	n.d.
	IBT 9393	n.d.	0.03 ± 0.001	2.0 ± 0.04
	IBT 9397	n.d.	46 ± 3	33 ± 0.5
	IBT 41107	n.d.	5.4 ± 1	6.7 ± 0.6
F. verticillioides	IBT 9400	n.d.	0.035 ± 0.005	35 ± 0.7
	IBT 9492	n.d.	0.028 ± 0.004	4.9 ± 0.2
	IBT 9496	n.d.	0.033 ± 0.0003	n.d.
	IBT 9502	n.d.	2.2 ± 0.4	18 ± 1
	IBT 9505	n.d.	0.078 ± 0.09	9.5 ± 3
	IBT 41110	n.d.	0.12 ± 0.07	4.5 ± 0.7
F. dlaminii	IBT 2937	n.d.	n.d.	n.d.
	IBT 2938	n.d.	n.d.	n.d.
F. napiforme	IBT 2931	n.d.	0.24 ± 0.06	6.2 ± 0.9
	IBT 2932	n.d.	0.13 ± 0.1	0.081 ± 0.02
F. nygamai	IBT 2933	n.d.	0.033 ± 0.006	n.d.
	IBT 2934	n.d.	22 ± 2	5.6 ± 0.6
	IBT 8290	n.d.	0.041 ± 0.006	0.047 ± 0.001
	IBT 8554	n.d.	0.033 ± 0.01	0.039 ± 0.003
	IBT 8557	n.d.	0.01 ± 0.01	0.14 ± 0.002
	IBT 8566	n.d.	3.1 ± 1	6.2 ± 0.5

Table 1: Fumonisin production by *Aspergillus niger* and *Fusarium* spp. on CYAS, PDA and RC after 7 days growth at 25°C. *(Continued)*

	IBT 9394	n.d.	0.30 ± 0.08	n.d.
	IBT 9395	n.d.	0.03 ± 0.003	16 ± 0.6
F. oxysporum	IBT 9514	n.d.	2.8 ± 0.4	37 ± 0.8

The concentration of FB_2 was detected in the methanol:water (3:1) extract. The values are means of the replicates plus/minus the standard deviation. The replicates are made in biological duplicates on two separate plates.
n.d. not detected.
Standard deviation calculated on two measurements

measured. Only seven strains had a total production of fumonisin above 1 μg/ml extract on RC compared to 14 strains on PDA.

Seven *Fusarium* strains did not have any measurable production of FB_1, FB_2 nor FB_3 on PDA compared to two non-producers on RC. On the other hand PDA supported production of higher amounts of fumonisins, strains which had a barely detectable amount of FB_1 on RC, also showed production of either FB_2 or both FB_2 and FB_3 on PDA.

Strains for the next experiments were selected on the basis of the above mentioned experiments. Besides the five *A. niger* strains five *Fusarium* strains were selected, with both good and poor producers at 25°C, two strains with a high FB production, *F. verticillioides* IBT 9400 and *F. oxysporum* IBT 9514, one with an average FB production *F. proliferatum* IBT 41107, one with a low FB_1 production *F. napiforme* IBT 2932 and a strain with a higher production of FB_2 than FB_1 *F. nygamai* IBT 8554 was selected.

The effect of temperature on growth and production of fumonisins by A. niger *and* Fusarium *spp*

Only one of the *A. niger* strains was able to grow at 15°C, although very slowly (extensive data shown in [Additional file 1]). The growth increased at higher temperature

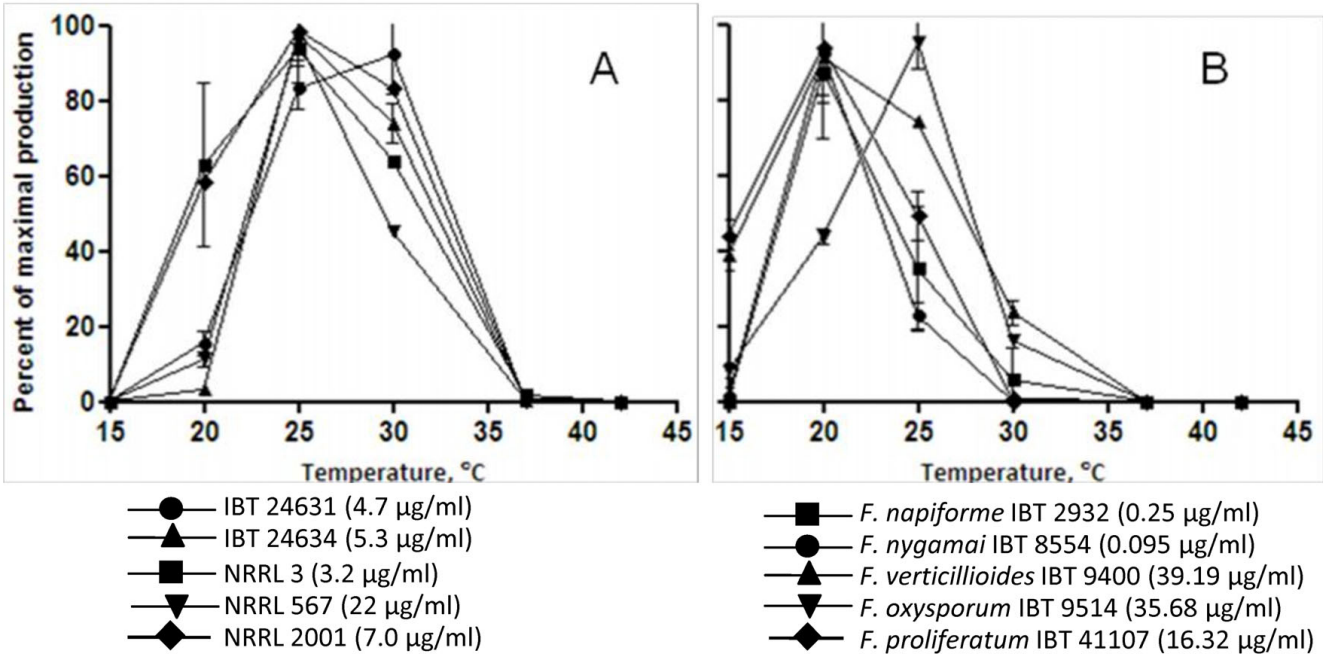

Figure 1

A: FB_2 production by *Aspergillus niger* after 7 days of growth at different temperatures (15-42°C) on CYAS. The concentration of FB_2 was detected in the methanol:water (3:1) extract. The values are means of biological duplicates on two different plates; highest value (μg/ml) is in parenthesis. B: Total production of fumonisin B_1, B_2 and B_3 produced by *Fusarium* spp. after 7 days growth at different temperatures (15-42°C) on PDA. The concentration of fumonisin was detected in the methanol:water (3:1) extract. The values are means of biological duplicates on two different plates; highest value (μg/ml) is in parenthesis.

and peaked at 30-37°C, followed by a slight reduction of the growth at 42°C. All *Fusarium* strains were able to grow at 15°C followed by an increased growth at higher temperatures and peaked at 25-30°C, above this temperature the growth decreased and no growth was observed at 42°C. The effect of temperature on the production of FB_2 by the five *A. niger* strains is shown in figure 1A. None of the isolates produced detectable amounts of FB_2 at 42°C, even though all strains grew well. The only *A. niger* strain, NRRL 2001, that was able to grow at 15°C, did not have any detectable production of FB_2.

At 37°C, the optimal temperature for growth by *A. niger*, the FB_2 production was very limited. NRRL 567 had the highest production of the five strains at 25°C and 30°C; however at 20°C NRRL 2001 showed the highest production. This strain showed also the highest growth rate at this temperature. Four strains, NRRL 567, NRRL 3, NRRL 2001 and IBT 24634 had the highest FB_2 production at 25°C, followed by 30°C, 20°C, and 37°C (Figure 1A). One strain, IBT 24631, differed from the other four, since it had a maximal production at 30°C, followed by 25°C, 20°C and 37°C.

The concentration of FB_1, FB_2 and FB_3 produced by *Fusarium* spp. is shown in figure 1B, and four of the *Fusarium* spp. had maximal production at 20°C: *F. verticillioides* IBT 9400, *F. proliferatum* IBT 41107, *F. napiforme* IBT 2932 and *F. nygamai* IBT 8554. *F. oxysporum* IBT 9514 had a maximal production of fumonisin at 25°C. Only three of the *Fusarium* strains, *F. verticillioides* IBT 9400, *F. oxysporum* IBT 9514 and *F. proliferatum* IBT 41107 had a measurable production of fumonisin at 15°C. *F. napiforme* IBT 2932 was only able to produce detectable amounts of fumonisins in the temperature range 20-25°C. At 20°C this strain had detectable concentrations of FB_1, FB_2 and FB_3, but at 25°C only FB_1 and FB_2 were detected (data not shown). Even though there was growth of all five *Fusarium* strains at 37°C there were only in one case detectable production of fumonisins, this was produced by *F. verticillioides*.

The effect of glycerol, NaCl and sucrose on the growth and production of fumonisins by A. niger *and* Fusarium *spp*

All strains of *A. niger* and *Fusarium* spp. were able to grow at all glycerol concentration (0-255 g/l) [see Additional file 2]. The growth of *A. niger* was only slightly reduced at a_w 0.99; below this there was a continuous decrease in the growth. For the *Fusarium* spp. the growth was reduced at a_w 0.99 and below, which was the same as the *A. niger*. All *A. niger* strains were able to produce FB_2 at all glycerol concentrations tested (Figure 2). The effect of glycerol on the quantitative FB_2 production for *A. niger* was very strain dependent, but in general the average FB_2 production was reduced 8.2% per 0.01 a_w unit (R^2 = 0.97). Three of the five *Fusarium* strains were able to produce fumonisin at all glycerol concentrations: *F. nygamai* IBT 8554, *F. oxysporum* IBT 9514 and *F. verticillioides* IBT 9400 (Figure 2). For two of these, IBT 8554 and IBT 9400, fumonisin production was increased up to 20% when glycerol was added. The other two strains *F. napiforme* IBT 2932 and *F. nygamai* IBT 8554 did not have a measurable production of fumonisins at a_w 0.99 and 0.98. The average total fumonisin production was reduced 18% per 0.01 a_w unit (linear regression R^2 = 0.91).

For the five *A. niger* growth in the presence of NaCl was partially inhibited below a_w 0.98 and all strains were able to grow at all a_w tested. The *Fusarium* species had the highest growth rates at the highest a_w and the growth declined until a_w 0.93 where they were unable to grow.

The FB_2 production of all *A. niger* strains was stimulated when NaCl was added with FB_2 being detected at all NaCl concentrations (Figure 2). The production peaked at a_w 0.985-0.97 depending on the strain. A reduction of the FB_2 production started at a_w 0.97-0.94 and a comparison by a regression analysis to the cultures with no NaCl added the average production of FB_2 decreased 13% per 0.01 a_w unit (R^2 = 0.98). The addition of NaCl clearly reduces the production of fumonisins by *Fusarium* (Figure 2), and was not detected in any culture below a_w 0.955. One strain, *F. napiforme* IBT 2932, did not even have a measurable production of fumonisin when NaCl was added. But no correlations in the average decrease of fumonisin production was observed.

The *A. niger* and *Fusarium* spp. strains grew better on sucrose, compared to NaCl and glycerol. The addition of sucrose increased the production of FB_2 for all the *A. niger* strains (Figure 2). The increase in the FB_2 production was followed by either a reduction or stagnation in the production level at higher sucrose concentrations. The average decrease in the total fumonisin production were 8.3% per 0.01 a_w unit (R^2 = 0.87)

Only three of the five *Fusarium* strains were able to produce FB_1, FB_2 and FB_3 when cultured on different sucrose concentrations (Figure 2). Because a commercial potato extract without sucrose was not obtainable, the base potato extract was prepared on home-made boiled potatoes. The three *Fusarium* strains with a detectable fumonisin production were *F. oxysporum* IBT 9514, *F. proliferatum* IBT 41107 and *F. verticillioides* IBT 9400. *Fusarium oxysporum* IBT 9514 had a maximal production at a_w 0.995, while *F. proliferatum* IBT 41107 peaked at a_w 0.999 and *F. verticillioides* IBT 9400 peaked at a_w 0.999. A reduction of the fumonisin production was observed at higher sucrose concentrations. There were no correlations in the average decrease of fumonisin production

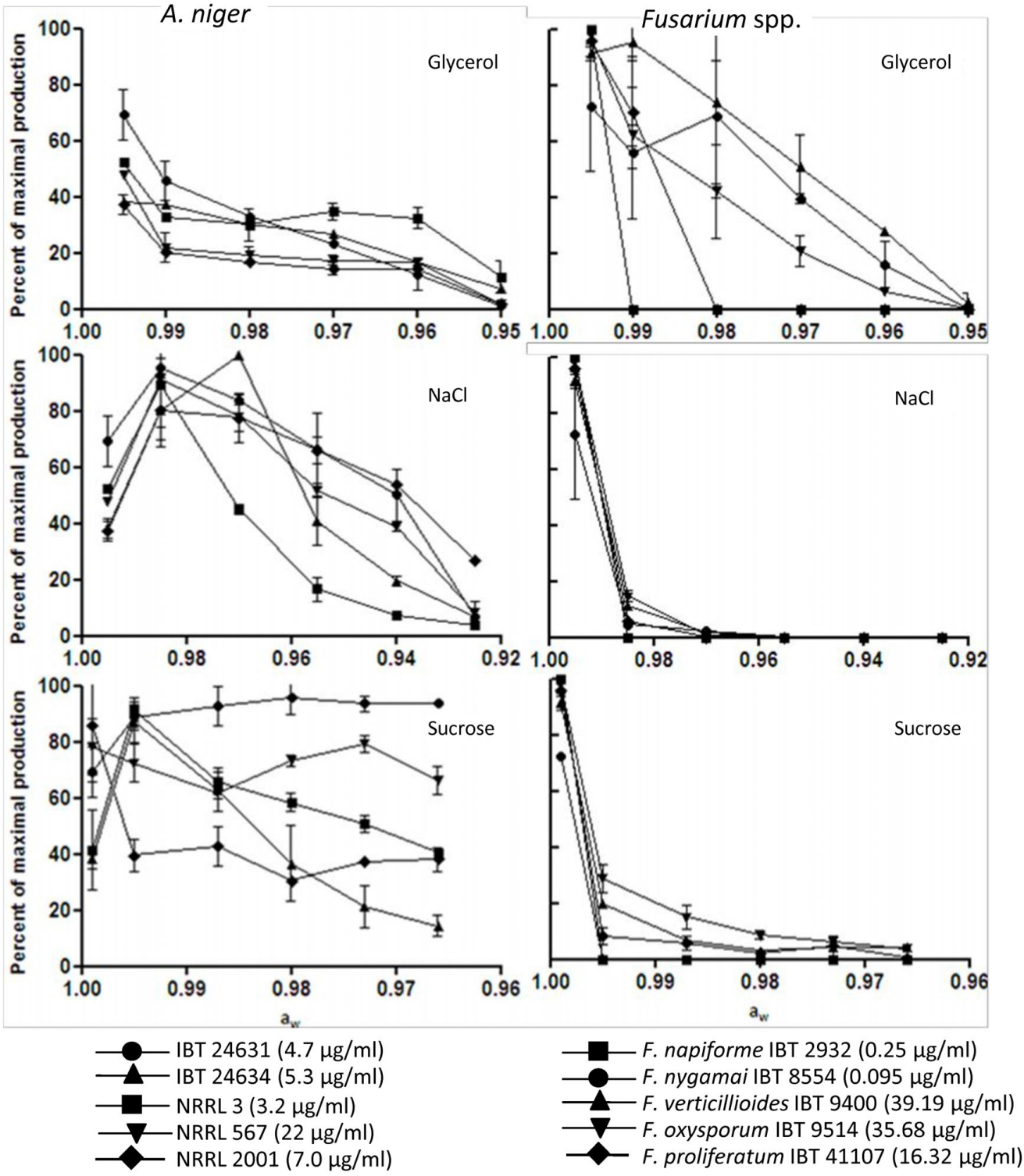

Figure 2

Effect of glycerol, NaCl and sucrose on the FB_2 production of *Aspergillus niger* and the total production of FB_1, FB_2 and FB_3 by *F. verticillioides, F. proliferatum*, *F. napiforme*, *F. nygamai* and *F. oxysporum*. Strains have been incubated at 25°C for 7 days on CYA or PDA. The concentration of fumonisins were detected in the methanol:water (3:1) extract. The values are means of biological duplicates on two different plates, highest value is in parenthesis.

Discussion

We found that *A. niger* was able to produce FB_2, in agreement with Frisvad *et al*. [34] who showed that *A. niger* produced FB_2 on the agar substrates RC and CYAS. On the other hand they did not measure any production of FB_2 on PDA, whereas all five *A. niger* strains in the present study had a detectable, albeit low, production of FB_2 on this substrate. Besides *F. verticillioides*, also *F. napiforme*, *F. nygamai*, *F. proliferatum* and *F. oxysporum* were found to produce fumonisins on laboratory agar substrates. The ability to produce fumonisins by these species correlates with findings of Nelson *et al*. [8] and Kpodo *et al*. [9]. However Nelson *et al*. [8] described the production of FB_1 by *F. dlaminii*, but this was not supported in our study. The findings of strains, capable of producing more FB_2 than FB_1 was also described by Musser & Plattner [36] as well as Leslie *et al*. [37]. Apart from the four *A. niger* strains shown to produce FB_2 by Frisvad *et al*. [34], additional 12 strains did produce this mycotoxin. Among the 18 *A. niger* strains investigated until now, only two have been unable to produce fumonisins in detectable amounts on the media investigated.

Astoreca *et al*. [38] found the optimal temperature for growth of *A. niger* to be 30°C, the highest investigated temperature in their study. In correlation to this study Palacios-Cabrera *et al*. [39] also found that *A. niger* grew optimally at temperatures of 30°C, which was also the optimal temperature for linear growth in our study. Marin *et al*. [40] found that the growth of both *F. verticillioides* and *F. proliferatum* was best at 25 to 30°C, which is in agreement of our results. According to Marin *et al*. [40]*F. verticillioides* is more tolerant to temperature above 30°C than other *Fusarium* spp., however this was not obvious in our study.

We found that the optimal production of FB_2 by *A. niger* was at 25°C and in one case 30°C. Since there has been only one report of the fumonisin production by *A. niger* we have compared our results to the production of ochratoxin A. Our results correlates with an investigation of Esteban *et al*. [41], who showed that the optimal temperature for production of ochratoxin A by *A. niger* was at 20-25°C. In contrast to this, other authors found that the optimal ochratoxin A production in a synthetic grape juice medium was significantly better at 15°C compared to both 25 and 35°C [42]. Findings in our study showed that at 20°C a significant decrease in the FB_2 production occurred compared to 30°C. Earlier studies show that the optimal temperature for production of fumonisins by *F. proliferatum* is at 15-20°C where *F. verticillioides* prefers the higher temperatures of 30°C [27]. This partly correlates with the results from our study, where both isolates showed the highest production at 20°C, but also produced fumonisins at 30°C. However fumonisin production by *F. verticillioides* was less inhibited than *F. proliferatum* by the higher temperature. Dilkin *et al*. [26] and Alberts *et al*. [25] found the optimal temperature for fumonisin production to be 25°C, followed by 20 and then 30°C. These results deviate from our results, because four isolates had the best production at 20°C and one at 25°C. Marin *et al*. [27] described the production of FB_1 by *F. verticillioides* at 37°C, which was also observed in our study. From our results a general pattern in the fumonisin production for both genera was observed, namely the maximal production of fumonisins being 5°C below the optimal growth.

Comparing *A. niger* with the *Fusarium* spp., all *A. niger* strains grew better at all tested a_W values. Leong *et al*. [42] found the optimal a_w for ochratoxin A production by *A. niger* to be a_w 0.95, whereas Esteban *et al*[43] found it to be in the range of 0.96-0.99, and that it was very strain dependent. These values are lower than those observed in our study for fumonisins where four of the *A. niger* strains had the highest production of FB_2 at a_W 0.99, with one isolate produced most FB_2 at a_w 0.98. Earlier studies have shown that the optimal a_W for fumonisin production by *Fusarium* is in the interval 0.97-0.98 [27,44]. The optimal a_w value from our study was a_W 0.995, which is a bit higher than the above mentioned a_w values. Frisvad *et al*. [34] also found that the addition of 5% NaCl (a_w = 0.97) or 20% sucrose (a_w = 0.99) increased the production of FB_2 by *A. niger*. The present study showed that not all the used strains had the same pattern. Only four of the five strains had an increase in FB_2 production at a_w 0.97 compared to the zero sample when grown on NaCl. The last *A.niger* NRRL 3 had only an increase at a_w 0.985 followed by a decrease at higher NaCl concentrations. The same was observed with sucrose where three strains had an increase in the FB_2 production compared to a sucrose concentration of 3% in standard medium. The last strains had a decrease in the production at the previous mentioned sucrose concentrations. In conclusion it is clear that there is very large strain variability in fumonisin production at different water activities between the *A. niger* strains used in these experiments.

Further studies on the effect of physiological variables on fumonisin production by *A. niger* is needed on large numbers of strains, due to large strain differences, and should further be backed up by studies of commodities where *A. niger* is common. This will lead us to a better understanding of how large a food safety problem fumonisin production by *A. niger* is. Only one report of fumonisins from *A. niger* in food (green coffee beans) has been reported, however the amounts were well below the regulatory limit [35]. Fumonisin contamination in a series of foods, often contaminated with *A. niger*, may show to be an overlooked mycotoxin problem, since surveys have only

mostly targeted corn and rice, based on the belief that it was only *Fusarium* spp. that could produce these important mycotoxins.

Conclusion

The present study shows that the regulation of fumonisin production is very different in *A. niger* compared to *Fusarium*, with the latter preferring high a_w (>0.99) and low temperature (20-25°C) and with *A. niger* preferring lower a_w and higher temperature (25-30°C). Fumonisin produced by *A. niger* may be an overlooked health risk in foods not previously associated with fumonisins, especially because *A. niger* is known as a common food spoilage organism on a wide variety of foods [45].

Methods

Unless otherwise is stated all solvents were HPLC grade, chemicals were analytical grade and water was purified on a Milli-Q system (Millipore, Bedford, MA). Media were prepared in 9 cm Petri dishes, each with 20 ml medium, and strains were inoculated by single point and dishes incubated in micro perforated plastic bags at 25°C for 7 days in darkness. The colony diameter measured is an average of the smallest and broadest diameter. All samples were as a minimum made in biological duplicates on two individually plates.

Fungal strains and media

All strains (Table 2) were from the IBT culture collection at Center for Microbial Biotechnology, DTU. The *Fusarium* strains were selected from species known to produce fumonisin. Five *A. niger* strains were selected, for the physiologically study, three of these have been used in industry according to collection databases (NRRL 3, NRRL 567 and NRRL 2001), whereas the last two were isolated from black pepper (IBT 24634, IBT 24631). The last 9 strains were only used for validation. All *A. niger* strains were characterized and identified using a polyphasic approach, as in our previous articles [46,47], in order to ensure that they were *A. niger sensu stricto*. Besides this the ITS region and parts of the β-tubulin and calmodulin genes were amplified and sequenced as described previously [48-50]. Results shown in Table 2 (public database numbers of the strains).

The media used for fumonisin production were: potato dextrose agar (PDA) [51], Czapek yeast autolysate agar with 5% NaCl (CYAS) [52] and rice meal corn steep liquor (RC) agar [53].

Fumonisin analysis

The fumonisin were extracted using the method previously described by Frisvad *et al*. [34]. Six plugs (D = 6 mm) were cut out of the colony from the center and in a radius towards the edge of the colony and transferred to a clean 2-ml vial, 800 μl of methanol:water (3:1) was added, and extracted by ultrasonication for one hour. All extracts were filtered through a 13 mm PTFE 0.45 μm syringe filter (National Scientific, Rockwood, Tennessee) into a new vial and used directly for analysis.

The LC-MS analysis was performed on a LC/MSD VL single quadrupole (Agilent, Santa Clara, California). The separation of 3 μl extracts were done at 40°C on a 50 × 2 mm, i.d. 3 μm size, Luna C-18 (II) column (Phenomenex, Torrance, California), fitted with a security guard column, and using a water:acetonitrile (both containing 20 mM formic acid) gradient at a flow rate of 0.3 ml/min. The gradient started at 30% acetonitrile, and increased to 60% acetonitrile over 5 minutes. During further 1 minute it was increased to 100% acetonitrile and maintained here for 2 minutes before the gradient in 1 minute was returned to starting conditions and kept there for 5 minutes. The mass spectrometer (MS) was operated in positive electrospray ionization mode and was automatically calibrated on the instrument ESI tuning mix. The MS was used in selected ion monitoring (SIM) mode for measuring $[M+H]^+$: FB_1 (m/z 722), FB_2 and FB_3 (m/z 706). The capillary voltage was held at 3000 V, the fragmentor voltage was at 70 V and the nebulizer pressure was at 2.5 bar. The drying gas flow was 12 l/min with a temperature of 350°C. The detection limit was measured to 0.01 μg/ml from dilutions of a FB_1 and FB_2 certified standard (Biopure, Tulin, Austria), with concentrations of 50.2 μg/ml and 51.0 μg/ml, respectively. The fumonisin concentrations of the extracts were calculated from a standard curve created from dilutions of the FB_1 and FB_2 standard mixture. FB_2 presence was further confirmed in selected extracts by LC-MS/MS [35] and LC-HRMS [34].

Efficiency of extraction solvents

The five different extraction solvents tested on *A. niger* NRRL 567 and *F. verticillioides* IBT 9400 were: *i*) methanol:water (3:1), *ii*) acetonitrile:water (3:1), *iii*) water at room temperature (25°C), *iv*) water at 100°C and *v*) methanol:dichloromethane:ethyl acetate (1:2:3) with addition of 1% (v/v) formic acid. The extraction process for [*i-iv*] was the same as mentioned above. For the fifth extraction solvent there were a few extra steps: After ultrasonication the extract was transferred to a new vial and the organic phase was evaporated *in vacuo*. The residue was redissolved by ultrasonication in 500 μl methanol for 20 minutes. All extracts were filtered through a PTFE 0.45 μm syringe filter before analysis.

Validation of methanol:water extraction

FB_2 extraction was validated by spiking 5 plugs of two non-fumonisin producing strains of *Aspergillus niger* (IBT 19345 and IBT 20381) with 100 μl FB_2 standard containing 5000, 2500, 1000, 500 and 100 ng FB_2. After spiking,

Table 2: Fungal isolates used for fumonisin production.

Fungi	Isolate	Genbank numbers
Aspergillus niger	NRRL 3 (ex unknown) (= ATCC 9069, CBS 120.49, IBT 23539) (Full genome sequenced)	FJ639289
	NRRL 567 (ex unknown) (= ATCC 12846, IBT 26387)	GU195638
	NRRL 2001 (ex unknown) (= ATCC 13794, IBT 26392)	GU195639
	IBT 24631(ex black pepper)	GU195636
	IBT 24634 (ex black pepper)	GU195637
Only used for validation	IBT 4983 (ex unknown) (= CBS 117.80)	GU195632
	IBT 18741 (ex carpet dust)	FJ639294
	IBT 19345 (ex unknown) (= IFO 6082)	GU195633
	IBT 19558(ex coffee beans)	GU195634
	IBT 20381 (ex coffee beans)	GU195635
	IBT 26774 (ex unknown)	
	IBT 28086 (ex grape)	
	IBT 28104 (ex black pepper)	GU105640
Fusarium dlaminii	IBT 2937 (ex plant debris) (= FRC M-1688)	
	IBT 2938 (ex plant debris) (= FRC M-1638)	
F. napiforme	IBT 2931 (ex soil debris from grassland) (= FRC M-1647)	
	IBT 2932 (ex soil debris from grassland) (= FRC M-1646)	
F. nygamai	IBT 2933 (ex unknown) (= FRC M-2376)	
	IBT 2934 (ex root debris from grassland) (= FRC M-2371)	
	IBT 8290 (ex unknown) (= MRC 4373)	
	IBT 8554 (ex unknown)	
	IBT 8557 (ex unknown)	
	IBT 8566 (ex corn kernel)	
	IBT 9394 (ex unknown) (= MRC 3997)	
	IBT 9395 (ex unknown) (= MRC 3998)	
F. oxysporum	IBT 9514 (ex corn kernel)	
F. proliferatum	IBT 8904 (ex yellow onion)	
	IBT 9109 (ex barley)	
	IBT 9337(ex corn stalk)	
	IBT 9393 (ex unknown) (= MRC 3218)	
	IBT 9397 (ex unknown) (= MRC 3216)	
	IBT 41107 (ex corn)	
F. verticillioides	IBT 9400 (ex unknown) (= MRC 826)	
	IBT 9492 (ex corn kernel)	
	IBT 9496 (ex corn kernel)	
	IBT 9502 (ex corn kernel)	
	IBT 9505 (ex corn kernel)	
	IBT 41110 (ex corn)	

ATCC: American Type Culture Collection, Manassas, VA, USA
CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
FRC: Fusarium Research Center, Penn State University, University Park, Pennsylvania, USA
IFO: Institute for Fermentation, Osaka, Japan.
MRC: South African Medical Research Council, Tygerberg, South Africa.
NRRL: Northern Regional Research Laboratory, Peoria, Illinois, USA.

Table 3: The concentration of glycerol, NaCl and sucrose and the corresponding measured water activity (a_w)

Glycerol (g/l)	0	51	102	154	204	255	---
a_w	1 ± 0	0.99 ± 0.0015	0.98 ± 0	0.97 ± 0.001	0.96 ± 0.001	0.95 ± 0.001	---
NaCl (g/l)	0	25	50	75	100	125	---
a_w	1 ± 0	0.985 ± 0.0006	0.97 ± 0.001	0.955 ± 0.0006	0.94 ± 0.0006	0.92 ± 0.0015	---
Sucrose (g/l)	0	30	130	230	330	430	530
a_w	1 ± 0	0.999 ± 0.0006	0.995 ± 0.0015	0.987 ± 0.0015	0.98 ± 0	0.973 ± 0.001	0.967 ± 0.001

The values are means of the triplicates plus/minus the standard deviation.

the plugs were left for 2 hours, and extracted as described above. Recovery was determined by comparing the slope of the spiked curve to slope of the curve from diluted samples in methanol-water (3:1). Five blank samples of the 2 strains were also analysed.

Reproducibility of extraction efficiency was further determined from 7 *A. niger* strains (IBT 4983, IBT 18741, IBT 19558, IBT 26774, IBT 28086, IBT 28104, NRRL 567) selected from high, medium and low fumonisin producing strains. Five replicate plates were made from each strain and extracted as described above.

The effect of temperature on the growth and production of fumonisin by A. niger *and* Fusarium *spp*

To assess the effect of temperature on the production of fumonisins, *A. niger* strains were inoculated on CYAS and *Fusarium* strains were inoculated on PDA. The plates were incubated in darkness at 15, 20, 25, 30, 37 or 42°C respectively for 7 days. The fungi used were the five *A. niger* strains listed in Table 2, and the following *Fusarium* species: *F. napiforme* IBT 2932, *F. nygamai* IBT 8554, *F. verticillioides* IBT 9400, *F. oxysporum* IBT 9514 and *F. proliferatum* IBT 41107.

The effect of glycerol, NaCl and sucrose on the growth and production of fumonisin by A. niger and Fusarium spp

For investigation of the effect of glycerol, NaCl and sucrose on the production of fumonisin the following experiments were performed: *A. niger* was inoculated on CYA and *Fusarium* on PDA with different concentration of glycerol (0-255 g/l), salt (0-125 g/l) and sucrose (0-530 g/l). For the *Fusarium*-sucrose experiment, the PDA mixture was made from boiled potatoes instead of a commercial blend. In short terms, 200 g potatoes was peeled and diced and autoclaved at 121°C with 1 liter of water, 15 g agar and 0-530 g sucrose.

The corresponding measured water activity (a_W) values of the media are shown in Table 3. The water activity was measured with an Aqualab (ADAB Analytical Devices, Stockholm, Sweden). There were no measurable differences in the water activity of the CYA and PDA media. The fungi used were five *A. niger* strains, and the following *F. napiforme* IBT 2932, *F. nygamai* IBT 8554, *F. verticillioides* IBT 9400, *F. oxysporum* IBT 9514 and *F. proliferatum* IBT 41107.

Authors' contributions

JMM, UT and JCF designed the study. JMM performed the experiments, KFN the analytical part and RAS the molecular genetic studies. All authors contributed in drafting the paper and have read and approved the final manuscript.

Additional material

Additional file 1

Effect of temperature on the growth of* Aspergillus niger *and* Fusarium *spp*. The conidial diameter of 5* Fusarium *spp. and 5* Aspergillus niger *strains at different temperature in the range of 15-42°C after 7 days growth.*

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Additional file 2

Effect of water activity on the growth of* Aspergillus niger *and* Fusarium *spp*. The conidial diameter of 5* Fusarium *spp. and 5* Aspergillus niger *strains at different a_w in the range of 0.92-1 after 7 days growth.*

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Acknowledgements

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References

1. Bezuidenhout SC, Gelderblom WCA, Gorst-Allman CP, Horak RM, Marasas WFO, Spiteller G, *et al.*: **Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*.** *J Chem Soc Chem Commun* 1988, **11:**743-745.
2. Branham BE, Plattner RD: **Isolation and characterization of a new fumonisin from liquid cultures of *Fusarium moniliforme*.** *J Nat Prod* 1993, **56:**1630-1633.
3. Musser SM, Gay ML, Mazzola EP, Plattner RD: **Identification of a new series of fumonisins containing 3-hydroxypyridine.** *J Nat Prod* 1996, **59:**970-972.
4. Ross PF, Rice LG, Osweiler GD, Nelson PE, Richard JL, Wilson TM: **A review and update of animal toxicoses associated with fumonisin contaminated feeds and production of fumonisins by *Fusarium i* solates.** *Mycopathologia* 1992, **117:**109-114.
5. Thiel PG, Shephard GS, Sydenham EW, Marasas WFO, Nelson PE, Wilson TM: **Levels of fumonisin B_1 and fumonisin B_2 in feeds associated with confirmed cases of equine leukoencephalomalacia.** *J Agric Food Chem* 1991, **39:**109-111.
6. Seifert KA, Aoki T, Baayen RP, Brayford D, Burgess LW, Chulze S, *et al.*: **The name Fusarium moniliforme should no longer be used.** *Mycol Res* 2003, **107:**643-644.
7. Gelderblom WCA, Jaskiewicz K, Marasas WFO, Thiel PG, Horak RM, Vleggaar R, *et al.*: **Fumonisins - Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*.** *Appl Environ Microbiol* 1988, **54:**1806-1811.
8. Nelson PE, Plattner RD, Shackelford DD, Desjardins AE: **Fumonisin B_1 production by *Fusarium s* pecies other than *F. moniliforme* in Section *Liseola* and by some related species.** *Appl Environ Microbiol* 1992, **58:**984-989.
9. Kpodo K, Thrane U, Hald B: **Fusaria and fumonisins in maize from Ghana and their co-occurrence with aflatoxins.** *Int J Food Microbiol* 2000, **61:**147-157.
10. Glenn AE: **Mycotoxigenic *Fusarium* species in animal feed.** *Anim Feed Sci Technol* 2007, **137:**213-240.
11. Sydenham EW, Shephard GS, Thiel PG, Marasas WFO, Stockenstrom S: **Fumonisin contamination of commercial corn-based human foodstuffs.** *J Agric Food Chem* 1991, **39:**2014-2018.
12. Harrison LR, Colvin BM, Greene JT, Newman LE, Cole JR Jr: **Pulmonary edema and hydrothorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium moniliforme*.** *J Vet Diagn Invest* 1990, **2:**217-221.
13. Soriano JM, Gonzalez L, Catala AI: **Mechanism of action of sphingolipids and their metabolites in the toxicity of fumonisin B_1.** *Prog Lipid Res* 2005, **44:**345-356.
14. Wang E, Norred WP, Bacon CW, Riley RT, Merrill AH: **Inhibition of sphingolipid biosynthesis by fumonisins - Implications for diseases associated with *Fusarium moniliforme*.** *J Biol Chem* 1991, **266:**14486-14490.
15. U.S.Food and Drug Administration: **Guidance for Industry: Fumonisin levels in human foods and animal feeds.** United States Food and Drug Administration, Washington DC; 2001.
16. EEC: **Setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products.** *Volume 1126*. Comm Eur Communities; 2007:14-16.
17. Stack ME: **Analysis of fumonisin B_1 and its hydrolysis product in tortillas.** *J AOAC Int* 1998, **81:**737-740.
18. Scott PM, Lawrence GA: **Analysis of beer for fumonisins.** *J Food Prot* 1995, **58:**1379-1382.
19. Park JW, Choi SY, Hwang HJ, Kim YB: **Fungal mycoflora and mycotoxins in Korean polished rice destined for humans.** *Int J Food Microbiol* 2005, **103:**305-314.
20. Martins ML, Martins HM, Bernardo F: **Fumonisins B_1 and B_2 in black tea and medicinal plants.** *J Food Prot* 2001, **64:**1268-1270.
21. Logrieco A, Doko B, Moretti A, Frisullo S, Visconti A: **Occurrence of fumonisin B_1 and B_2 in *Fusarium proliferatum* infected asparagus plants.** *J Agric Food Chem* 1998, **46:**5201-5204.
22. Marin S, Ramos AJ, Vazquez C, Sanchis V: **Contamination of pine nuts by fumonisin produced by strains of *Fusarium proliferatum* isolated from *Pinus pinea*.** *Lett Appl Microbiol* 2007, **44:**68-72.
23. Nelson PE, Juba JH, Ross PF, Rice LG: **Fumonisin production by *Fusarium* species on solid substrates.** *J AOAC Int* 1994, **77:**522-525.
24. Vismer HF, Snijman PW, Marasas WFO, van Schalkwyk DJ: **Production of fumonisins by *Fusarium verticillioides* strains on solid and in a defined liquid medium - Effects of L-methionine and inoculum.** *Mycopathologia* 2004, **158:**99-106.
25. Alberts JF, Gelderblom WCA, Thiel PG, Marasas WFO, van Schalkwyk DJ, Behrend Y: **Effects of temperature and incubation period on production of fumonisin B_1 by *Fusarium moniliforme*.** *Appl Environ Microbiol* 1990, **56:**1729-1733.
26. Dilkin P, Mallmann CA, de Almeida CAA, Stefanon EB, Fontana FZ, Milbradt EL: **Production of fumonisins by strains of *Fusarium moniliforme* according to temperature, moisture and growth period.** *Braz J Microbiol* 2002, **33:**111-118.
27. Marin S, Magan N, Belli A, Ramos AJ, Canela R, Sanchis V: **Two-dimensional profiles of fumonisin B_1 production by *Fusarium moniliforme* and *Fusarium proliferatum* in relation to environmental factors and potential for modelling toxin formation in maize grain.** *Int J Food Microbiol* 1999, **51:**159-167.
28. Samapundo S, Devliehgere F, De Meulenaer B, Debevere J: **Effect of water activity and temperature on growth and the relationship between fumonisin production and the radial growth of *Fusarium verticillioides* and *Fusarium proliferatum* on corn.** *J Food Prot* 2005, **68:**1054-1059.
29. Keller SE, Sullivan TM, Chirtel S: **Factors affecting the growth of *Fusarium proliferatum* and the production of fumonisin B_1: oxygen and pH.** *J Ind Microbiol Biotechnol* 1997, **19:**305-309.
30. Shim WB, Woloshuk CP: **Nitrogen repression of fumonisin B_1 biosynthesis in *Gibberella fujikuroi*.** *FEMS Microbiol Lett* 1999, **177:**109-116.
31. Branham BE, Plattner RD: **Alanine is a precursor in the biosynthesis of fumonisin B_1 by *Fusarium moniliforme*.** *Mycopathologia* 1993, **124:**99-104.
32. Baker SE: ***Aspergillus niger* genomics: Past, present and into the future.** *Med Mycol* 2006, **44:**S17-S21.
33. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, *et al.*: **Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88.** *Nat Biotechnol* 2007, **25:**221-231.
34. Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U: **Fumonisin B_2 production by *Aspergillus niger*.** *J Agric Food Chem* 2007, **55:**9727-9732.
35. Noonim P, Mahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA: **Fumonisin B_2 production by *Aspergillus niger* from Thai coffee beans.** *Food Addit Contam* 2009, **26:**94-100.
36. Musser SM, Plattner RD: **Fumonisin composition in cultures of *Fusarium moniliforme*, *Fusarium proliferatum*, and *Fusarium nygami*.** *J Agric Food Chem* 1997, **45:**1169-1173.
37. Leslie JF, Zeller KA, Lamprecht SC, Rheeder JP, Marasas WFO: **Toxicity, pathogenicity, and genetic differentiation of five species of *Fusarium* from sorghum and millet.** *Phytopathology* 2005, **95:**275-283.
38. Astoreca A, Magnoli C, Ramirez ML, Combina M, Dalcero A: **Water activity and temperature effects on growth of *Aspergillus niger, A. awamori* and *A. carbonarius* isolated from different substrates in Argentina.** *Int J Food Microbiol* 2007, **119:**314-318.
39. Palacios-Cabrera H, Taniwaki MH, Hashimoto JM, de Menezes HC: **Growth of *Aspergillus ochraceus*, *A. carbonarius* and *A. niger* on culture media at different water activities and temperatures.** *Braz J Microbiol* 2005, **36:**24-28.
40. Marin S, Sanchis V, Magan N: **Water activity, temperature, and pH effects on growth of *Fusarium moniliforme* and *Fusarium proliferatum* isolates from maize.** *Can J Microbiol* 1995, **41:**1063-1070.
41. Esteban A, Abarca ML, Bragulat MR, Cabañes FJ: **Effects of temperature and incubation time on production of ochratoxin A by black aspergilli.** *Res Microbiol* 2004, **155:**861-866.
42. Leong SLL, Hocking AD, Scott ES: **Effect of temperature and water activity on growth and ochratoxin A production by Australian *Aspergillus carbonarius* and *A. niger* isolates on a simulated grape juice medium.** *Int J Food Microbiol* 2006, **110:**209-216.
43. Esteban A, Abarca ML, Bragulat MR, Cabañes FJ: **Effect of water activity on ochratoxin A production by *Aspergillus niger* aggregate species.** *Int J Food Microbiol* 2006, **108:**188-195.
44. Etcheverry M, Torres A, Ramirez ML, Chulze S, Magan N: **In vitro control of growth and fumonisin production by *Fusarium verticillioides* and *F. proliferatum* using antioxidants under different water availability and temperature regimes.** *J Appl Microbiol* 2002, **92:**624-632.

45. Pitt JI, Hocking AD: *Fungi and food spoilage* 2nd edition. London, U.K.: Blackie Academic and Professional; 1997.
46. Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J: **Diagnostic tools to identify black aspergilli.** *Stud Mycol* 2007, **59:**129-145.
47. Samson RA, Houbraken JAMP, Kuijpers AFA, Frank JM, Frisvad JC: **New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*.** *Stud Mycol* 2004, **50:**45-61.
48. Varga J, Kocsube S, Toth B, Frisvad JC, Perrone G, Susca A, *et al.*: ***Aspergillus brasiliensis* sp. nov., a biseriate black *Aspergillus* species with world-wide distribution.** *Int J Syst Evol Micr* 2007, **57:**1925-1932.
49. Varga J, Frisvad JC, Samson RA: **Polyphasic taxonomy of *Aspergillus* section *Candidi* based on molecular, morphological and physiological data.** *Stud Mycol* 2007, **59:**75-88.
50. Varga J, Due M, Frisvad JC, Samson RA: **Taxonomic revision of *Aspergillus* section *Clavati* based on molecular, morphological and physiological data.** *Stud Mycol* 2007, **59:**89-106.
51. Samson RA, Hoekstra ES, Frisvad JC: *Introduction to food and airborne fungi* 7th edition. Utrecht, the Netherlands: Centralbureau voor Schimmelcultures; 2004.
52. Frisvad JC, Samson RA: **Polyphasic taxonomy of *Penicillium* subgenus *Penicillium* - A guide to identification of food and airborne terverticillate Penicillia and their mycotoxins.** *Stud Mycol* 2004, **49:**1-173.
53. Bullerman LB: **Screening medium and method to detect several mycotoxins in mold cultures.** *J Milk Food Technol* 1974, **37:**1-3.

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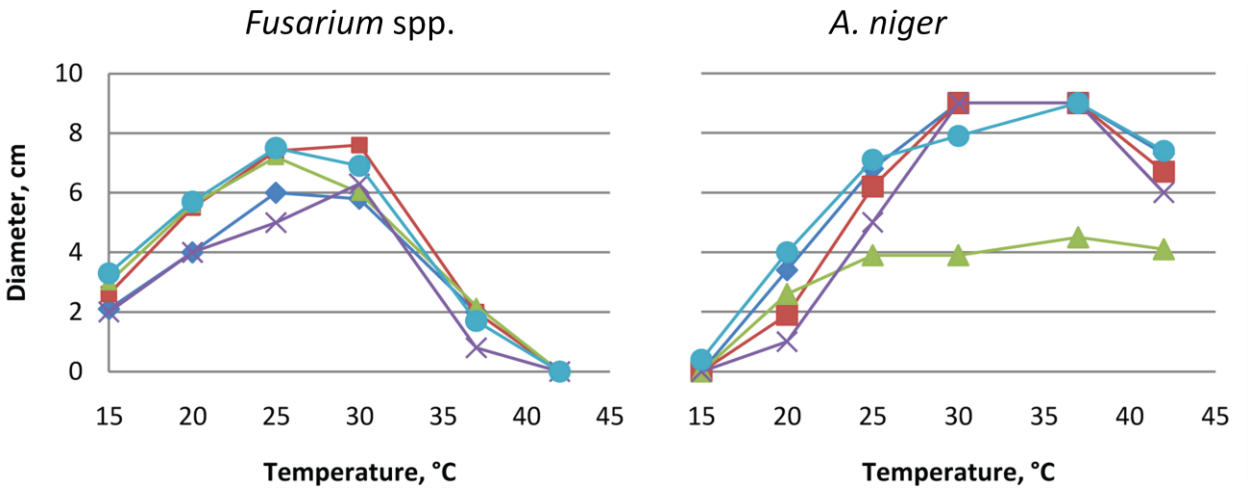
Additional file 1

The conidial diameter of 5 *Fusarium* spp. and 5 *Aspergillus niger* isolates at different temperature in the range of 15-42 °C. All isolates were grown in perforated plastic bags at 25°C for 7 days in darkness. The diameter is an average of the smallest and broadest conidial diameter.

F. napiforme 2932 *F. nygamai* 8554 *F. verticillioides* 9400 *F. oxysporum* 9514 *F. proliferatum* 41107

A. niger : NRRL 3 NRRL 567 NRRL 2001 IBT 24631 IBT 24634

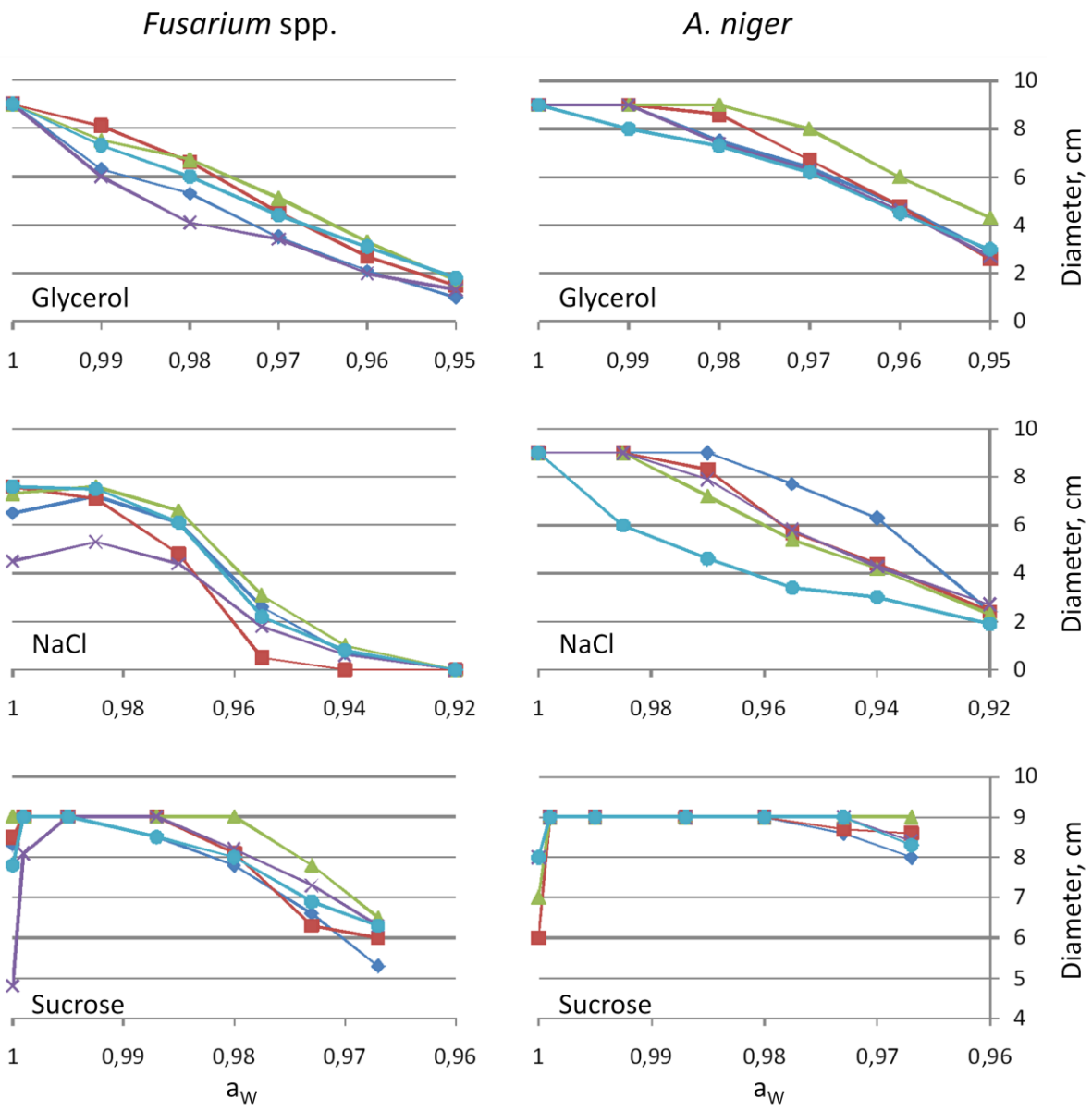


Additional file 2

The conidial diameter of 5 *Fusarium* spp. and 5 *Aspergillus niger* isolates on three different water activity lowering solutes, glycerol, NaCl and sucrose. A_w was in the range of 0.92-1. All isolates were grown in perforated plastic bags at 25°C for 7 days in darkness. The diameter is an average of the smallest and broadest conidial diameter.

F. napiforme 2932 *F. nygamai* 8554 *F. verticillioides* 9400 *F. oxysporum* 9514 *F. proliferatum* 41107

A. niger : NRRL 3 NRRL 567 NRRL 2001 IBT 24631 IBT 24634



Paper 2

" Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins"

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Production of Fumonisin B_2 and B_4 by *Aspergillus niger* on Grapes and Raisins

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The recent discovery of fumonisin production in *Aspergillus niger*, raises concerns about the presence of these mycotoxins in grapes and raisins as well as other commodities where *A. niger* is a frequent contaminant. Here we investigate the potential production of fumonisins in *A. niger* cultured on grapes and raisins. Sixty-six *A. niger*, 4 *A. tubingensis*, and 16 *A. acidus* strains isolated from raisins were tested for fumonisin production on laboratory media. Neither *A. tubingensis* nor *A. acidus* strains produced fumonisins, but 77% of *A. niger* strains did. None of the strains produced ochratoxin A. Ten selected fumonisin producing *A. niger* strains were further able to produce fumonisin B_2 and fumonisin B_4 on grapes in the range 171−7841 μg fumonisin B_2/kg and 14−1157 μg fumonisin B_4/kg. Four selected strains were able to produce fumonisin B_2 (5−6476 μg/kg) and fumonisin B_4 (12−672 μg/kg) on raisins.

KEYWORDS: Fumonisins; *Aspergillus niger*; mycotoxins; grapes; raisins.

INTRODUCTION

Black Aspergilli are the most common fungi responsible for postharvest decay of fresh fruit (*1*). Of these *Aspergillus carbonarius* and *A. niger* are very important opportunistic pathogens of grapes causing bunch rot or berryrot and causing raisin mold (*2*). Since *A. carbonarius* is a much better ochratoxin A producer, the main focus concerning grapes and black Aspergilli has been on *A. carbonarius* and ochratoxin A (*3*). However, *A. niger* has also been reported to grow and damage a large number of crops and foods worldwide, including corn, peanuts, raisins, onions, mangoes, apples, and dried meat products (*1*).

A. niger is used for production of single cell protein for feed (*4*) and is extensively used for production of several organic acids and extracellular enzymes. Furthermore it is used as a transformation host in the biotechnological industry (*5*, *6*). The discovery of putative homologues to the *Fusarium verticillioides* fumonisin gene cluster in three *A. niger* genomes led to the discovery of a fumonisin B_2 production by all three full genome sequenced strains and the ex type culture of *A. niger* (*6*−*8*). It has also been shown that *A. niger* is able to produce the biosynthetic precursor fumonisin B_4, which lacks a hydroxyl group on the backbone (*9*). The structure of fumonisins B_1, B_2, and B_4 are shown in **Figure 1**.

Fumonisins are important mycotoxins because they are suspected to cause human and animal toxicoses by the consumption of contaminated corn-based food and feeds (*10*). The fumonisins are structurally similar to sphingolipids and have shown to inhibit the sphingolipid biosynthesis via the ceramide synthase pathway (*11*). Fumonisins have shown to induce outbreaks of leukoencephalomalacia in horses and pulmonary edema and hydrothorax in pigs (*11*, *12*). Fumonisin B_1 is hepatocarcinogenic, hepatotoxic, and nephrotoxic in rats and rat liver cancer can be promoted and initiated by fumonisin B_1 (*11*, *12*). Fumonisin B_1 and B_2 have been declared class 2B carcinogens, which are possible human carcinogens (*13*, *14*). The regulatory limit for fumonisins in corn is, according to the U.S. Food and Drug Administration, 2−4 μg/g total fumonisins (*15*). EEC has a regulatory limit of 0.2−2 μg/g (*16*). Fumonisins have been isolated from corn and corn-based products (*12*) such as tortillas (*17*) and beer (*12*), as well as rice (*12*), black tea leaves (*18*), asparagus (*19*), and pine nuts (*20*). Very few factors that affect the fumonisin production by *A. niger* have been investigated; so far only temperature and water activity and a limited amount of media have been investigated (*8*, *21*). Recently fumonisins B_2 and B_4 from *A. niger* were detected in green coffee beans, although the concentrations (up to 10 μg/kg) were well below the regulatory limit for other commodities (*9*). In a study performed on grape must, 2 out of 12 samples were found to be positive for fumonisin B_2 (0.01 and 0.4 μg/g)(*22*).

In this study, we investigate the presence of ochratoxin A and fumonisin producing black Aspergilli on raisins, as well as fumonisin production in grapes, dried grapes, and raisins.

MATERIALS AND METHODS

Unless otherwise is stated all solvents were HPLC grade, chemicals were analytical grade, and water was purified on a Milli-Q (Millipore, Billerica, MA). Media were prepared in 9 cm Petri dishes, each with 20 mL of medium. The fumonisin standard was a mixed certified standard containing both fumonisin B_1 and fumonisin B_2 with concentrations of 50.2 and 51.0 mg/L (Biopure, Tulln, Austria). The ochratoxin A standard contained 10 mg/L (Biopure). Fungal strains were three-point inoculated and all samples were incubated in microperforated plastic bags at 25 °C for 7 days in darkness. All water activity measurements were

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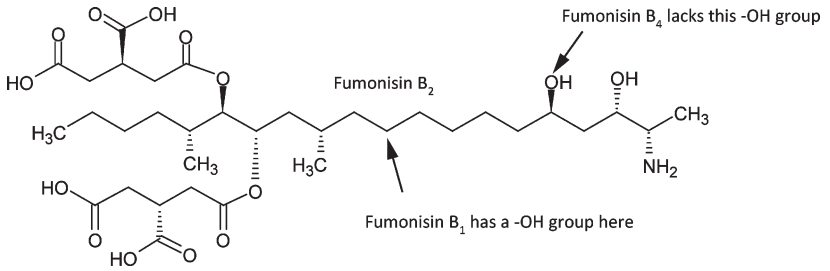

Figure 1. Fumonisin B_2 and the Difference to Fumonisin B_1 and B_4.

performed in triplicate and measured with an Aqualab (ADAB Analytical Devices, Stockholm, Sweden).

Isolation and Characterization of Black Aspergilli Isolated from Raisins. Seventeen different raisin samples were purchased from supermarkets and shops in Denmark, U.S.A., or Uganda. The raisins originated from California, Argentina, or Uganda. Six raisins were selected randomly from each sample, placed on a dichloran 18% glycerol agar (*23*) plate, and incubated at 25 °C for seven days. All black Aspergilli were transferred to Czapek yeast autolysate agar (CYA) and yeast extract sucrose agar (YES) (*24*) plates and afterward identified using a polyphasic approach (macro- and micromorphology and metabolite profile) according to previous reports (*25*−*27*). All strains were subsequently screened for a potential fumonisin production on three different media, CYA, YES, and Czapek yeast autolysate agar with 5% NaCl (CYAS) (*24*). Fumonisins and ochratoxin A were extracted using an agar plug extraction method (*8*) with small modifications. Six agar plugs (D = 6 mm) were transferred to a 2 mL vial and 800 μL of methanol/water (3:1) was added. The samples were extracted ultrasonically for 1 h, filtered through a PFTE 0.45 μm filter (National Scientific, Rockwood, TN), and used directly for LC-TOF-MS analysis. Representative isolates were preserved in the IBT collection (author's address).

Preparation of Grapes. Conidial suspensions for inoculation of grapes were obtained by harvesting spores of each isolate grown on CYA and suspending them in sterile distilled water containing 0.05% of Tween 80 (Merck, Hohenbrunn, Germany) and 0.05% agar (Bie & Berntsen, Rødovre, Denmark). The final concentration of the conidia was assessed by using a counting chamber and was adjusted to 10^6 conidia/mL.

Fresh grapes (Thompson seedless), cut in half, were placed in an empty Petri dish and inoculated with 10 μL of the *A. niger* conidia suspensions. The grape experiment was prepared in duplicate. Ten strains randomly selected from the before mentioned screening were selected for the grape experiment, IBT 28747, 28753, 28934, 28937, 28948, 28964, 28965, 28966, 28994, and 29019.

Preparation of Commercial Raisins. From one brand, two subsamples of 125 g raisins (California, Thompson seedless) were randomly selected and surface sterilized with hypochlorite (3%) for 15 min and then washed twice with sterile water (weight gain 7%). One of the two subsamples was covered with water and both subsamples were kept at 5 °C for 24 h (weight gain 50%). The raisins placed in water were transferred to an empty Petri dish; the other subsample was placed on a water agar separated by a filter (pore size 1 μm). Four *A. niger* strains with the highest production of fumonisins in grapes were chosen; these were IBT 28753, 28934, 28948, and 29019. The raisins were inoculated with 10 μL of the *A. niger* conidia suspensions in quadruplicates as described above.

Preparation of Dried Grapes. Portions of 300 g grapes (size, 2−3 g) (Thompson seedless) were taken at random and dried in an oven at 75 °C for 4 h (weight loss, 32%, initial a_w 0.76 ± 0.04), 5 h (weight loss, 38%, initial a_w 0.69 ± 0.01), and 6.5 h (weight loss, 52%, initial a_w 0.54 ± 0.09). Subsequently, they were placed in an empty Petri dish and inoculated with *A. niger* as described above. All samples were as a minimum prepared in triplicates. Four *A. niger* strains with the highest production of fumonisins in grapes were chosen; these were IBT 28753, 28934, 28948, and 29019.

Extraction of Grapes and Raisins for Chemical Analysis. One grape or raisin was weighed and placed in a 5 mL cryo tube with 5−10 steel balls (D = 3 mm), 5 mL methanol/water (3:1) was added. The cryo tubes were shaken in a Mini Beadbeater 96 (Biospec Product Inc., Bartlesville, OK) for 5 min. The whole mixture was transferred to a 15 mL Falcon tube. Afterward it was placed on a shaking table (at an angle of 45°) for 60 min at 150 rpm. The Falcon tubes were centrifuged at 8000 g for 4 min and 200 μL extract was transferred to a 2 mL HPLC vial and used directly for LC-MS/MS analysis. Because of nonavailability of fumonisin B_4 as analytical standard, external quantification was performed with fumonisin B_2 and similar response factors were assumed for the two mycotoxins.

LC-TOF-MS Conditions. The screening for a fumonisin in culture extracts production was performed using a LC system coupled to an orthogonal TOF mass spectrometer (Micromass LCT, Manchester, U.K.) equipped with an electrospray source (*25*). The column used was a 50 × 2 mm i.d., 3 μm Gemini C_6-phenyl column (Phenomenex, Torrance, CA) with a linear gradient starting from 30% acetonitrile in water (both 20 mM formic acid) to 60% acetonitrile for 5 min at a flow rate of 300 μL/min, which was then increased to 100% acetonitrile in 1 min and a flow of 0.5 mL/min, keeping this for 3.5 min before returning to the start conditions in 6 min. The mass spectrometry was performed in ESI^+.

LC-MS/MS Conditions. LC-MS/MS analysis was performed as previously described (*9*) with an Agilent HP 1100 liquid chromatography system (Waldbronn, Germany) coupled to a Quattro Ultima triple mass spectrometer (Micromass, Manchester, UK) with ESI source. The gradient was as described before but with the gradient starting at 20% CH_3CN and going to 55% CH_3CN in 5 min and then to 100% in 30 s. Tandem mass spectrometry was performed in ESI^+ at a source flow at 700 L/h nitrogen at 350 °C. Nitrogen was also used as collision gas, and the MS operated in multiple reaction monitoring mode at the following transitions: fumonisin B_2 quantifier m/z 706 → 336 cone 50 V, collision 40 V, dwell time 50 ms, qualifier m/z 706 → 512, cone 50 V, collision 25 V, dwell time 100 ms (ion ratio, 1.7−2.1 (transition 1/2)); fumonisin B_4 quantifier m/z 690 → 320 cone 50 V, collision 35 V, dwell time 50 ms, qualifier m/z 690 → 514 a, cone 50 V, collision 30 V, dwell time 100 ms (ion ratio, 1.4−1.8 (transition 1/2)); ochratoxin A quantifier m/z 404→ 239, cone 30 V, collision 33 V, dwell time 100 ms, qualifier m/z 404 → 358, cone 30 V, collision 25 V, dwell time 100 ms (ion ratio, 1.4−1.7 (transition 1/2)).

Validation. The method was validated by spiking raisins and grapes with 100 μL of a mixture of fumonisin B_2 and ochratoxin A standards to the following six different final levels: fumonisin B_2, 15, 25, 50, 150, 200, and 300 μg/kg; ochratoxin A, 3, 5, 10, 30, 40, and 60 μg/kg in triplicate. The samples were left to dry for 2 h prior to extraction. Both the raisins and the grapes were extracted as described above.

RESULTS AND DISCUSSION

Preliminary testing (results not shown) of strong anion exchange purification (SAX) showed low or no recoveries of fumonisins from the grapes, presumably due to competition from high amounts organic acids from the grapes and/or from *A. niger* strains themselves. Furthermore immunoaffinity purification (results not shown) yielded viscous brown extracts from raisin extracts and was thus not suitable either. Consequently, it was decided to analyze the crude extracts.

For validation, standard curves were prepared and shown to be linear. On grapes, the relative standard deviation of the lowest point (15 μg/kg) was 18% (fumonisin B_2 level); for raisins it was 7%. All R^2, level of detection (LOD), and level of quantification (LOQ) values are shown in **Table 1**. Because of the high fumonisin concentrations detected in the strains, LOD_{fum} were not

experimentally addressed, but estimation from the lowest point down to s/n indicates LOD of 5 μg/kg. Control analysis of conidial suspensions shows that in approximately 10 000 spores the concentration of fumonisin was in picogram levels and would be diluted 1:500 in the final extract thus not influencing the result.

The screening resulted in isolation of 86 black Aspergilli strains; of these 66 were *A. niger*, 4 *A. tubingensis*, and 16 *A. acidus* (= *A. foeditus* var. *acidus* (*28*)). None of the *A. tubingensis* and *A. acidus* strains produced fumonisins, but 77% of the *A. niger* produced fumonisin B_2 (LC-TOF). Similar results were found among a limited number of grape derived strains (*22*). A similar recent study performed on green coffee beans found the a similar fraction (76%) of *A. niger* as fumonisin B_2 producers (*9*). The percentage of ochratoxin A -producing *A. niger* is most often reported in the interval 0−30% (*3*, *29*−*31*), but in one case a percentage as high as 41% has been reported (*32*). The percentages of ochratoxigenic strains of *A. niger* are very low compared to our 77% fumonisin producing *A. niger*. Therefore this fumonisin production by *A. niger* could present an even larger food safety problem.

Table 1. R^2, Level of Determination (LOD), and Level of Quantification (LOQ) Determined from Spiked Raisin and Grape Samples

	fumonisin B_2			ochratoxin A		
matrix	R^2 (n = 18)	LOD[a]	LOQ	R^2 (n = 18)	LOD[b]	LOQ
grape	0.993	5 μg/kg	25 μg/kg	0.96	3 μg/kg	10 μg/kg
raisin	0.990	3 μg/kg	25 μg/kg			

[a] Estimated from the lowest point (15 μg/kg) to s/n (1:5). [b] The s/n is 1:5.

Table 2. Production of Fumonisins by *Aspergillus niger* in Grapes after 7 Days Growth at 25°C

isolate	fumonisin B_2 (μg/kg)		fumonisin B_4 (μg/kg)[a]		
IBT	sample 1	sample 2	sample 1	sample 2	ochratoxin A μg/kg[b]
28747	951	1283	75	189	nd
28753	7703	7979	772	1542	nd
28934	2098	5376	158	612	nd
28937	1045	1941	253	265	nd
28948	3380	4772	349	509	nd
28964	91	251	5	23	nd
28965	1074	2414	148	344	nd
28966	1261	5123	220	634	nd
28994	432	2422	36	158	nd
29019	4303	11189	143	405	nd

[a] Assuming same response factor as fumonisin B_2. LOD: 5 μg/kg. [b] LOD: 3 μg/kg. nd: not detected.

The 10 *A. niger* strains with the highest measured fumonisin production were selected for further experiments, IBT 28747, 28753, 28934, 28937, 28948, 28964, 28965, 28966, 28994, and 29019. All 10 strains were able to produce fumonisins in grapes (**Table 2**). Fumonisin B_2 was produced as the major component, followed by fumonisin B_4. The mean production of fumonisin B_2 varied almost 50 fold from 171 μg/kg (IBT 28964) to 7841 μg/kg (IBT 28753) and fumonisin B_4 varied almost 70 fold from 14 μg/kg (IBT 28964) to 1157 μg/kg (IBT 28753). Selected transitions from the LC-MS/MS analysis of infected and spiked grapes are shown in **Figure 2**. The measured fumonisin concentrations are similar, compared to those previously described by Logrieco et al. (200−2500 μg/kg) (*22*).

The four strains with the highest production of fumonisins on grapes were selected for the work with dried grapes and raisins. On the dried grapes, fumonisin B_2 and fumonisin B_4 were also produced (**Table 3**). The concentration of fumonisin B_2 was in the range of 91−1747 μg/kg and fumonisin B_4 9−69 μg/kg. The fumonisin production was highest in the grapes with the lowest weight losses and the lowest production was in all coincidences found in the grapes with the lowest water content.

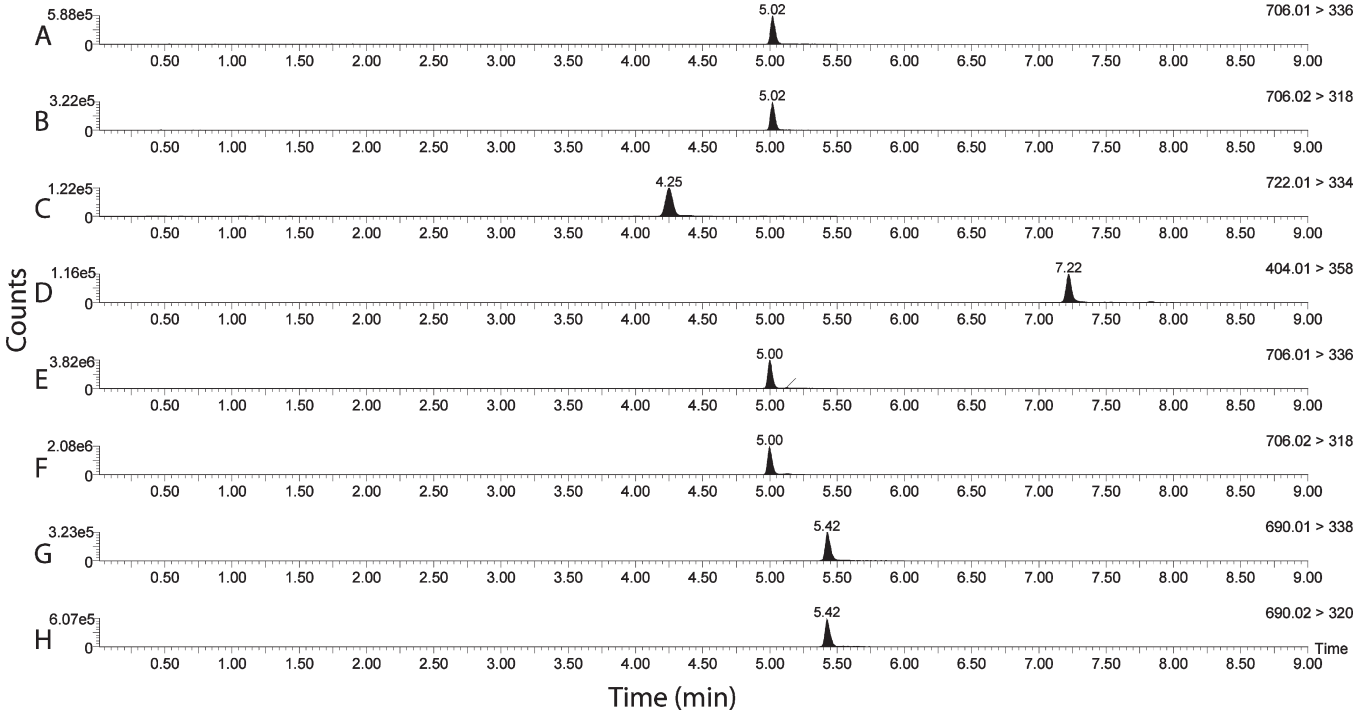

Figure 2. Results from the LC-MS/MS analysis. Transitions of Ochratoxin A. Fumonisin B_1. B_2 and B_4 in Spiked Grape and Infected Grape. (**A**) Quantifier of Fumonisin B_2 from a Spiked Grape (150 μg/kg). (**B**) Qualifier of Fumonisin B_2 from a Spiked Grape (150 μg/kg). (**C**) Quantifier of Fumonisin B_1 from a Spiked Grape (150 μg/kg). (**D**) Quantifier of Ochratoxin A from a Spiked Grape (30 μg/kg). (**E**) Quantifier of Fumonisin B_2 from an Infected Grape (7841 μg/kg IBT 28753). (**F**) Qualifier of Fumonisin B_2 from an Infected Grape (7841 μg/kg IBT 28753). (**G**) Quantifier of Fumonisin B_4 from an Infected Grape (1157 μg/kg IBT 28753). (**H**) Qualifier of Fumonisin B_4 from an Infected Grape (1157 μg/kg IBT 28753).

Table 3. Production of Fumonisins by *Aspergillus niger* in Dried Grapes after 7 Days Growth at 25°C

isolate IBT	weight loss %	initial a_w	fumonisin B_2 μg/kg[a]	fumonisin B_4 μg/kg[a,b]	ochratoxin A μg/kg[c]
28753	32	0.76 ± 0.04	1747 ± 316	48 ± 22	nd
	38	0.69 ± 0.01	1128 ± 329	48 ± 16	nd
	52	0.54 ± 0.09	91 ± 73	9 ± 4	nd
28934	32	0.76 ± 0.04	1363 ± 954	69 ± 51	nd
	38	0.69 ± 0.01	1033 ± 460	50 ± 23	nd
	52	0.54 ± 0.09	636 ± 397	24 ± 16	nd
28948	32	0.76 ± 0.04	313 ± 323	54 ± 47	nd
	38	0.69 ± 0.01	154 ± 71	27 ± 11	nd
	52	0.54 ± 0.09	144 ± 104	29 ± 19	nd
29019	32	0.76 ± 0.04	203 ± 106	25 ± 11	nd
	38	0.69 ± 0.01	214 ± 137	28 ± 13	nd
	52	0.54 ± 0.09	102 ± 76	10 ± 5	nd

[a] The values are means of the quadruplicates, plus/minus the standard deviation. [b] Assuming same response factor as Fumonisin B_2. LOD: 5 μg/kg. [c] LOD: 3 μg/kg. nd: not detected.

Table 4. Production of Fumonisins by *Aspergillus niger* in Raisins after 7 Days Growth at 25°C

isolate IBT	weight gain	initial a_w	fumonisin B_2 μg/kg[a]	fumonisin B_4 μg/kg[a,b]	ochratoxin A μg/kg[c]
28753	7% (agar)	0.77 ± 0.05	1160 ± 686	105 ± 56	nd
	50% (water)	0.95 ± 0.001	407 ± 249	47 ± 25	nd
28934	7% (agar)	0.77 ± 0.05	229 ± 58	27 ± 7	nd
	50% (water)	0.95 ± 0.001	5 ± 5	nd	nd
28948	7% (agar)	0.77 ± 0.05	459 ± 430	45 ± 31	nd
	50% (water)	0.95 ± 0.001	112 ± 51	12 ± 4	nd
29019	7% (agar)	0.77 ± 0.05	6476 ± 1139	356 ± 46	nd
	50% (water)	0.95 ± 0.001	784 ± 636	672 ± 422	nd

[a] The values are means of the quadruplicates, plus/minus the standard deviation. [b] Assuming same response factor as Fumonisin B2. LOD: 5 μg/kg [c] LOD: 3 μg/kg. nd: not detected.

Two experiments were carried out with the raisins, one where the water activity (a_w) initially was low and over time increased toward 1, and another where the water activity started high and decreased over time. All of the tested *A. niger* strains were found to produce fumonisin B_2 and fumonisin B_4 on both types of raisins (**Table 4**). In all cases the production of fumonisins was highest when the raisins were kept moist. Fumonisin B_2 and fumonisin B_4 were produced in the range of 229−6476 and 27−356 μg/kg. The raisins with a decreasing water activity had a fumonisin B_2 concentration of 5−784 μg/kg and fumonisin B_4 of 12−672 μg/kg.

In the dried grapes the production of fumonisins decreased as the water activity was lowered. This correlates with earlier results that the lower a_w, the more reduced fumonisin production by *A. niger* (*21*). The reason why the raisins with the lowest a_w supported fumonisin production could be because the wet grapes dried quickly and the raisins on the water agar were kept moist with a high a_w on the surface. For the four tested strains, in the three experiments all had the highest production of fumonisins in grapes.

This is the first report describing the production of fumonisins B_2 and B_4 by *A. niger* in dried grapes and raisins. All the *A. niger* produced more fumonisin B_2 than fumonisin B_4. No production of ochratoxin A (LOD: 3 μg/kg) was detected in either the grape or raisin experiments. This is supported by earlier studies that have shown *A. carbonarius* and not *A. niger* as the most important source of ochratoxin A in raisins (*29*, *33*, *34*).

The presence of a fumonisin production in grapes and a detectable content of fumonisin in grape must (*22*) indicates that fumonisins may be found in wine unless degraded during the fermentation or storage. Consequently, a larger number of wines as well as grape musts need to be investigated to track the fate of fumonisins in all production steps from grapes to wine.

Fumonisin produced by *A. niger* may be an overlooked health risk in a broad variety of foods, because *A. niger* is a common food spoilage organism (*1*). New knowledge within this field is important, particularly because corn and derived products are currently the only major products that are monitored for fumonisins.

LITERATURE CITED

(1) Pitt, J. I.; Hocking, A. D. *Fungi and food spoilage*, 2nd ed.; Blackie Academic and Professional: London, U.K., 1997.

(2) Varga, J.; Juhasz, A.; Kevei, F.; Kozakiewicz, Z. Molecular diversity of agriculturally important *Aspergillus* species. *Eur. J. Plant Pathol.* **2004**, *110*, 627–640.

(3) Cabañes, F. J.; Accensi, F.; Bragulat, M. R.; Abarca, M. L.; Castella, G.; Minguez, S.; Pons, A. What is the source of ochratoxin A in wine? *Int. J. Food Microbiol.* **2002**, *79*, 213–215.

(4) Singh, A.; Abidi, A. B.; Agrawal, A. K.; Darmwal, N. S. Single cell protein production by *Aspergillus niger* and its evaluation. *Zbl. Mikrobiol.* **1991**, *146*, 181–184.

(5) Schuster, E.; Dunn-Coleman, N.; Frisvad, J. C.; van Dijck, P. W. M. On the safety of *Aspergillus niger* - a review. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 426–435.

(6) Pel, H. J.; de Winde, J. H.; Archer, D. B.; Dyer, P. S.; Hofmann, G.; Schaap, P. J.; Turner, G.; de Vries, R. P.; Albang, R.; Albermann, K.; Andersen, M. R.; Bendtsen, J. D.; Benen, J. A. E.; van den Berg, M.; Breestraat, S.; Caddick, M. X.; Contreras, R.; Cornell, M.; Coutinho, P. M.; Danchin, E. G. J.; Debets, A. J. M.; Dekker, P.; van Dijck, P. W. M.; van Dijk, A.; Dijkhuizen, L.; Driessen, A. J. M.; d'Enfert, C.; Geysens, S.; Goosen, C.; Groot, G. S. P.; de Groot, P. W. J.; Guillemette, T.; Henrissat, B.; Herweijer, M.; van den Hombergh, J. P. T. W.; van den Hondel, C. A. M. J.; van der Heijden, R. T. J. M.; van der Kaaij, R. M.; Klis, F. M.; Kools, H. J.; Kubicek, C. P.; van Kuyk, P. A.; Lauber, J.; Lu, X.; van der Maarel, M. J. E. C.; Meulenberg, R.; Menke, H.; Mortimer, M. A.; Nielsen, J.; Oliver, S. G.; Olsthoorn, M.; Pal, K.; van Peij, N. N. M. E.; Ram, A. F. J.; Rinas, U.; Roubos, J. A.; Sagt, C. M. J.; Schmoll, M.; Sun, J. B.; Ussery, D.; Varga, J.; Vervecken, W.; de Vondervoort, P. J. J. V.; Wedler, H.; Wosten, H. A. B.; Zeng, A. P.; van Ooyen, A. J. J.; Visser, J.; Stam, H. Genome sequencing and analysis of the

versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.* **2007**, *25*, 221–231.
(7) Baker, S. E. *Aspergillus niger* genomics: Past, present and into the future. *Med. Mycol.* **2006**, *44*, S17–S21.
(8) Frisvad, J. C.; Smedsgaard, J.; Samson, R. A.; Larsen, T. O.; Thrane, U. Fumonisin B_2 production by *Aspergillus niger*. *J. Agric. Food Chem.* **2007**, *55*, 9727–9732.
(9) Noonim, P.; Mahakarnchanakul, W.; Nielsen, K. F.; Frisvad, J. C.; Samson, R. A. Fumonisin B_2 production by *Aspergillus niger* from Thai coffee beans. *Food Addit. Contam., Part A* **2009**, *26*, 94–100.
(10) Sydenham, E. W.; Shephard, G. S.; Thiel, P. G.; Marasas, W. F. O.; Stockenstrom, S. Fumonisin contamination of commercial corn-based human foodstuffs. *J. Agric. Food Chem.* **1991**, *39*, 2014–2018.
(11) Stockmann-Juvala, H.; Savolainen, K. A review of the toxic effects and mechanisms of action of fumonisin B_1. *Hum. Exp. Toxicol.* **2008**, *27*, 799–809.
(12) Yazar, S.; Omurtag, G. Z. Fumonisins, trichothecenes, and zearalenone in cereals. *Int. J. Mol. Sci.* **2008**, *9*, 2062–2090.
(13) IARC Toxins derived from *Fusarium moniliforme*: Fumonisins B_1 and B_2 and fusarin C. *IARC.* **1993**, *56*, 445.
(14) IARC Fumonisin B_1. *IARC Monogr.* **2002**, *82*, 301.
(15) U.S. Food and Drug Administration Guidance for Industry: Fumonisin levels in human foods and animal feeds. U. S. Food and Drug Administration: Washington, DC, 2001.
(16) EEC Setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. *Comm. Eur. Communities* **2007**, *1126*, 14−16.
(17) Stack, M. E. Analysis of fumonisin B1 and its hydrolysis product in tortillas. *J. AOAC Int.* **1998**, *81*, 737–740.
(18) Martins, M. L.; Martins, H. M.; Bernardo, F. Fumonisins B1 and B2 in black tea and medicinal plants. *J. Food Prot.* **2001**, *64*, 1268–1270.
(19) Logrieco, A.; Doko, B.; Moretti, A.; Frisullo, S.; Visconti, A. Occurrence of fumonisin B1 and B2 in *Fusarium proliferatum* infected asparagus plants. *J. Agric. Food Chem.* **1998**, *46*, 5201–5204.
(20) Marin, S.; Ramos, A. J.; Vazquez, C.; Sanchis, V. Contamination of pine nuts by fumonisin produced by strains of *Fusarium proliferatum* isolated from *Pinus pinea*. *Lett. Appl. Microbiol.* **2007**, *44*, 68–72.
(21) Mogensen, J. M.; Nielsen, K. F.; Samson, R. A.; Frisvad, J. C.; Thrane, U. Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* species. *BMC Microbiol.*, accepted.
(22) Logrieco, A.; Ferracane, R.; Haidukowsky, M.; Cozzi, G.; Visconti, A.; Ritieni, A. Fumonisin B2 production by *Aspergillus niger* from grapes and natural occurrence in must. *Food Addit. Contam., Part A* **2009**, *26*, 1495–1500.
(23) Hocking, A. D.; Pitt, J. I. Dichloran glycerol medium for enumeration of xerophilic fungi from low moisture foods. *Appl. Environ. Microbiol.* **1980**, *39*, 488–492.
(24) Frisvad, J. C.; Samson, R. A. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium* - A guide to identification of food and airborne terverticillate Penicillia and their mycotoxins. *Stud. Mycol.* **2004**, *49*, 1–173.
(25) Nielsen, K. F.; Mogensen, J. M.; Johansen, M.; Larsen, T. O.; Frisvad, J. C. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Anal Bioanal. Chem.* **2009**, *395*, 1225–1242.
(26) Samson, R. A.; Noonim, P.; Meijer, M.; Houbraken, J.; Frisvad, J. C.; Varga, J. Diagnostic tools to identify black aspergilli. *Stud. Mycol.* **2007**, *59*, 129–145.
(27) Samson, R. A.; Houbraken, J. A. M. P.; Kuijpers, A. F. A.; Frank, J. M.; Frisvad, J. C. New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. *Stud. Mycol.* **2004**, *50*, 45–61.
(28) Kozakiewicz, Z. *Aspergillus* species on stored products. *Mycol. Pap.* **1989**, *161*, 1–188.
(29) Abarca, M. L.; Accensi, F.; Bragulat, M. R.; Castella, G.; Cabanes, F. J. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *J. Food Prot.* **2003**, *66*, 504–506.
(30) Accensi, F.; Abarca, M. L.; Cano, J.; Figuera, L.; Cabañes, F. J. Distribution of ochratoxin A producing strains in the *A. niger* aggregate. *Anton. Leeuw. Int. J. G.* **2001**, *79*, 365–370.
(31) Romero, S. M.; Comerio, R. M.; Larumbe, G.; Ritieni, A.; Vaamonde, G.; Pinto, V. F. Toxigenic fungi isolated from dried vine fruits in Argentina. *Int. J. Food Microbiol.* **2005**, *104*, 43–49.
(32) Magnoli, C.; Violante, M.; Combina, M.; Palacio, G.; Dalcero, A. Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. *Lett. Appl. Microbiol.* **2003**, *37*, 179–184.
(33) Valero, A.; Marin, S.; Ramos, A. J.; Sanchis, V. Ochratoxin A-producing species in grapes and sun-dried grapes and their relation to ecophysiological factors. *Lett. Appl. Microbiol.* **2005**, *41*, 196–201.
(34) Magnoli, C.; Astoreca, A.; Ponsone, L.; Combina, M.; Palacio, G.; Rosa, C. A. R.; Dalcero, A. M. Survey of mycoflora and ochratoxin A in dried vine fruits from Argentina markets. *Lett. Appl. Microbiol.* **2004**, *39*, 326–331.

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Paper 3

" Widespread occurrence of the mycotoxin Fumonisin B_2 in wine"

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Widespread Occurrence of the Mycotoxin Fumonisin B_2 in Wine

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Fumonisins are important mycotoxins because they are suspected to cause human and animal toxicoses by the consumption of contaminated corn-based food and feeds. However, with the discovery of fumonisin production in grapes by *Aspergillus niger*, wine may also be a fumonisin-containing commodity. In the present study, we have developed a simple and quantitative cation-exchange-based purification method for the subsequent isotope dilution liquid chromatography−tandem mass spectrometry (LC−MS/MS) determination of fumonisins in wine. A comparative study of seven different solid-phase extraction (SPE) columns showed that polymeric mixed-mode reversed-phase (RP) cation-exchange columns were superior to classic silica-based cation and mixed-mode cation-exchange columns. A total of 77 wine samples from 13 countries were subsequently tested, and surprisingly, 18 (23%) were found to contain fumonisin B_2 in the range of 1−25 μg/L. These findings were further confirmed by immunoaffinity purification and re-analysis of the positive cation-exchanged extracts.

KEYWORDS: Fumonisin; wine; grapes; *Aspergillus niger*

INTRODUCTION

Black aspergilli are some of the most common fungi responsible for postharvest decay of fresh fruit (*1*). Of these are *Aspergillus carbonarius* and *Aspergillus niger*, very important opportunistic pathogens of grapes causing bunch rot or berryrot (*2*). Because *A. carbonarius* can produce large amounts of ochratoxin A, the main focus concerning grapes, wine, and black aspergilli has therefore been on this fungus and ochratoxin A (*3*−*6*). The recent discovery of fumonisins B_2 (*7*), B_4 (*8*), and B_6 (*9*) (**Figure 1**) from *A. niger* and the production of fumonisins by *A. niger* in coffee and especially grapes, raisins, and must (*8*, *10*, *11*) have raised concerns of the possible general presence of fumonisins in wine.

Fumonisins are important mycotoxins because they are suspected to cause human and animal toxicoses by the consumption of contaminated corn-based food and feeds (*12*). To avoid possible detrimental health effects, the United States Food and Drug Administration (U.S. FDA) recommends that maize should not be used for human consumption when contaminated with more than 2−4 mg/kg total fumonisins (*13*), whereas the European Union (EU) has a regulatory limit of 0.2−2 mg/kg (*14*).

Thus far, no detection method for fumonisins in wine has been described. Non-purified but highly concentrated extracts of must, grapes, and raisins have been analyzed using liquid chromatography−tandem mass spectrometry (LC−MS/MS) (*10*, *11*). However, for low concentrations in a high complexity of this product, wine (e.g., containing polyphenolics, flavonoids, tannins, and anthocyanins), this approach will clearly limit sensitivity and poses ion-suppression problems unless a much more sensitive instrument is used and, thus, less sample can be injected. We therefore decided to develop a fast solid-phase extraction method with subsequent LC−MS/MS analysis for the determination of fumonisins in wine. The method described here is to our knowledge the first quantitative method for fumonisins using cation exchange.

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MATERIALS AND METHODS

Unless otherwise stated, all solvents were high-performance liquid chromatography (HPLC)-grade and other chemicals were analytical-grade and from Sigma-Aldrich (St. Louis, MO). Water was purified on a Milli-Q system (Millipore, Bedford, MA). The fumonisin standard was a mixed certified standard containing both fumonisins B_1 and B_2, with concentrations of 50.2 and 51.0 mg/L (Biopure, Tulln, Austria). Relevant samples were added U-$^{13}C_{34}$-fumonisin B_2 (10.52 μg/mL, Biopure) to a total concentration of 10 μg/L.

Sample Preparation. Wine samples of 10 mL (spiked or non-spiked, pH∼3.5−4) were, unless otherwise stated, passed through a 30 mg/3 mL Strata X-C column (Phenomenex, Torrance, CA), which was previously conditioned with 1 mL of methanol and 1 mL of water. To further acidify the column, 1 mL of water with 2% formic acid was added. Afterward, the column was washed with 1 mL of methanol and the fumonisins were eluted with methanol/water (1:4) with 2% NH_4OH water into a 2 mL HPLC vial and used directly for analysis.

The following other cation-exchange columns were tested with the above method as well as small variations of this: Oasis MCX, 30 mg (Waters, Milford, MA) and the silica based ones, HCX, 130 mg (C_8, SCX, Biotage, Uppsala, Sweden); HCX-3, 130 mg (C_{18}, SCX, Biotage); HCX-5, 130 mg (C_4, SCX, Biotage); and SCX, 100 mg (Phenomenex).

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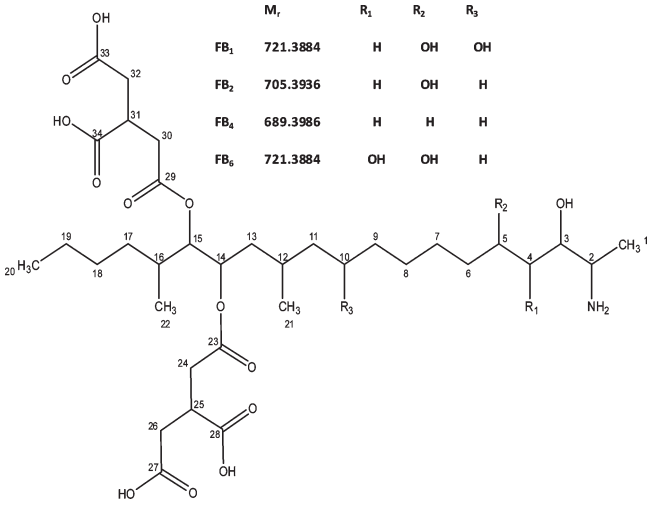

Figure 1. Structure of fumonisin B_2 and a comparison to fumonisins B_1, B_4, and B_6.

SAX purification was also tested on 100 mg columns (Phenomenex). The following procedure was used: The pH of the wine was adjusted to 7.0 ± 0.2 with 1M NaOH, and the columns were conditioned with 1 mL of methanol and 1 mL of water, followed by the addition of wine. The cartridge was washed with 1 mL of methanol/water (7:3) and 1 mL of methanol. The samples were eluted with methanol with 2% formic acid and used directly for analysis. Each sample was spiked with an internal standard of U-$^{13}C_{34}$-fumonisin B_2 to a total concentration of 10 μg/L.

Validation. The fumonisin B_2 extraction was validated by spiking portions of 10 mL of wine to the following concentrations of fumonisin B_2: 0 (blank), 2, 5, 15, 50, 150, and 450 μg/L. The experiments were performed on different days, with all levels in triplicates each day. The apparent recovery was calculated from methanol/water (20:80) diluted samples with the following concentrations of fumonisin B_2: 2, 5, 15, 50, 150, and 450 μg/L. An internal standard of U-$^{13}C_{34}$-fumonisin B_2 was added to each sample to a total volume of 10 μg/L. To determine the apparent recovery, the slope of spiked wine samples and MeOH standards was compared for similar concentrations.

Wine Samples. Wine samples for screening were either bought in a local supermarket or kindly provided by employees at the Department of Systems Biology, Technical University of Denmark. In total, 77 wines from 13 countries were tested, including red (n = 56), white (n = 13), port (n = 6), champagne (n = 1), rosé (n = 1), and madeira (n = 1) wines. The wines were from the years 1991−2009 and from Argentina (n = 1), Australia (n = 6), Chile (n = 3), China (n = 1), France (n = 22), Germany (n = 1), Italy (n = 23), New Zealand (n = 1), Portugal (n = 7), Romania (n = 1), Spain (n = 9), South Africa (n = 2), and the U.S. (California) (n = 1). All information on the wines was taken from the label of the bottle.

LC−MS/MS Conditions. LC−MS/MS analysis was performed as previously described (*11*) but with minor changes. Briefly described, the LC−MS/MS analysis was performed on a Quattro Ultima triple mass spectrometer (Micromass, Manchester, U.K.) with a z-spray electrospray ionization (ESI) source. Separations was performed on a 50 × 2 mm inner diameter, 3 μm Gemini C_6-phenyl column (Phenomenex, Torrance, CA). Using a linear gradient starting from 20% acetonitrile in water (both supplemented with 20 mM formic acid) to 55% acetonitrile for 6 min at a flow rate of 0.3 mL/min, which was then increased to 100% acetonitrile in 30 s at a flow of 0.5 mL/min, keeping this for 3.5 min before returning to the start conditions in 6 min. MS/MS was performed in ESI^+, and the MS operated in multi reaction monitoring (MRM) mode at the following transitions: fumonisin B_2, quantifier m/z 706 → 336, cone, 50 V; collision, 40 V; dwell time, 50 ms; qualifier m/z 706 → 512, cone, 50 V; collision, 25 V; dwell time, 100 ms; fumonisin B_4, quantifier m/z 690 → 320, cone, 50 V; collision, 35 V; dwell time, 50 ms; qualifier m/z 690 → 514, cone, 50 V; collision, 30 V; dwell time, 100 ms; fumonisins B_1 and B_6, quantifier m/z 722 → 334, cone, 50 V; collision, 40 V; dwell time, 50 ms; qualifier m/z 722 → 528, cone, 50 V; collision, 25 V; dwell time, 100 ms; and U-$^{13}C_{34}$-fumonisin B_2 m/z 740 → 358, cone, 50 V; collision, 50 V; dwell time, 50 ms.

Verification of Positive Samples. Because a significant number of the positive samples were close to the limit of quantitation (LOQ), it was decided to verify the findings by purification on FumoniTest immunoaffinity columns (Vicam, Watertown, MA). Half of the purified extract (500 μL) from the LC−MS/MS screening was diluted with phosphate-buffered saline (PBS) buffer to a final volume of 10 mL. The pH was adjusted to 7 ± 0.3 with 1 M HCl before it was loaded onto the immunoaffinity column (1−2 drops/s). The column was washed with 10 mL of PBS buffer; air was pushed through to remove buffer; and possible fumonisins were eluted with 1.5 mL of methanol (1 drop/s). The eluate was evaporated to dryness under a stream of nitrogen at approximately 45 °C, and the dried residue was redissolved in 100 μL of methanol/water (1:4) and re-analyzed by LC−MS/MS (5 μL injected).

RESULTS AND DISCUSSION

Sample Purification. Fumonisins have mainly been purified by anion-exchange or immunoaffinity columns (*15*, *16*). Our first approach was the anion-exchange approach using SAX columns, which could be used to target not only fumonisins but also ochratoxin A. However, SAX purification was irreproducible and suffered from poor recovery (≪50%), as also observed in a previous study on the analysis of fumonisins from grapes, presumably because of the competition from organic acids from the grapes (*11*). Subsequently, it was decided to use cation exchange, because fumonisins have an amine group, thereby avoiding the anionic compounds. In our first attempt, we compared Strata SCX (silica-based) to polymeric Strata X-C, with the latter being superior, because of stable flow and better recovery. To find an optimal column, we further compared five different mixed-mode reversed-phase (RP) cation-exchange SPE columns for their abilities to extract fumonisins from wine. The worst tested was clearly the anion-exchange column (SAX) with only 11% extracted fumonisin compared to the best column (MCX). Concerning the C_4, C_8, and C_{18} mixed-mode SCX columns, there was a recovery of 71, 50, and 12%, respectively, indicating that an increase in the hydrophobicity of the column resulted in a more inefficient recovery of fumonisins. Two of the six tested columns, Oasis MCX and Strata X-C, clearly gave recoveries of 86 and 83%, respectively, with the latter being slightly more reproducible.

To determine the loading capacity of fumonisins on the Strata X-C, seven concentrations within the range of 100−1500 μg/L fumonisin spiked wine were loaded onto columns and, after LC−MS/MS analysis, the eluted fumonisin showed a linear relationship with no signs of reaching a maximal capacity, indicating a maximal capacity above 1500 μg/L.

Validation. The three calibration curves, one from each day, from spiked samples, had the following R^2 values: 0.9928, 0.9957, and 0.9978, with a relative standard deviation (RSD) of the slope being 10%. The RSD of the spiked samples (N = 9) varied within (0.6−35%), with an average RSD of 15%. The lowest concentration (2 μg/L) gave a s/n of 20:1, indicating that the limit of detection (LOD) was approximately 4 times lower (assuming a s/n of 5:1). Recovery was found to be 60−93%, with an average of 83% and an average RSD of 12%.

Screening of Wine Samples. Analysis of the 77 wine samples demonstrated the occurrence of fumonisin B_2 in 23% of the samples (18 of 77). This method gives four identification points via retention time and two MRMs and, thus, exceeding the requirements for identification (three points) (*17*). Because this is the first report describing fumonisin B_2 in wine and that a high number of samples were close to the LOQ, it was decided to further confirm the results by immunoaffinity purification. This was not formally validated because the use of the ^{13}C standard validates each purification. By comparison of the peak areas of

Table 1. Overview of the Tested Wine Samples and Their Fumonisin Content[a]

sample number	country	grape sort	year	wine type	fumonisin (μg/L)
1	Australia	Chardonnay	2008	white	nd[b]
2	Spain	Monastrell		red	2.1
3	Australia	Shiraz, Cabernet Sauvignon	2006	red	nd
4	Italy			red	nd
5	South Africa	Cabernet Sauvignon	2007	red	2.5
6	Australia	Shiraz		rose	nd
7	Romania	Pinot Noir	2006	red	20
8	Spain	Tempranillo Garnacha	2007	red	nd
9	Italy	Merlot, Corvina	2007	red	7.0
10	Australia	Shiraz	2008	red	1.0
14	Italy	Corvina, Rondinella, Molinarea, Negrana	2007	red	6.7
17	France	Sauvignon Blanc, Semillion Muscadelle	2003	white	nd
18	Italy	Corvina, Rondinella	2007	red	nd
19	France	Chardonnay	2008	white	nd
20	France		2007	champagne	nd
21	Chile	Chardonnay	2008	white	nd
24	France	Carignan, Grenache, Syrah	2003	red	5.1
25	Italy		2005	red	nd
26	France	Chardonnay	2008	white	nd
27	Spain	Tempranillo, Gaunacha	2005	red	1.9
29	Italy	Shiraz	2007	red	nd
30	Italy		2003	red	nd
31	Italy		1998	red	nd
33	California, U.S.A.	Zinfandel	1998	red	25
34	Portugal		2007	port	nd
36	Italy	Grecanico, Chardonnay	2008	white	nd
37	France	Grenache, Syrah	2003	red	1.3
38	Portugal		2002	madeira	nd
39	Italy	Montepulciano, Aglianico	2005	red	2.1
40	Italy	Merlot, Corbina	2007	red	3.9
42	Chile	Chardonnay	2008	white	nd
43	Australia		2008	white	nd
44	South Africa		2008	white	nd
45	France	Grenache, Syrah	2007	red	nd
46	Italy	Malvasia, Trebbiano	2008	white	nd
47	Italy	Montalcino, Sangiovese Grosso, Brunello	2000	red	nd
49	Italy	Nebbiolo	1996	red	nd
50	Australia	Shiraz	2007	red	nd
51	China	Cabernet Sauvignon	2007	red	nd
52	Germany	Pinot Noir	2004	red	nd
53	France	Cabernet Sauvignon, Cabernet Franc, Merlot, Petit Verdot	1999	red	nd
54	France		1998	red	nd
57	New Zealand	Sauvignon Blanc	2008	white	nd
58	Italy	Negroamaro, Primitivo	2006	red	1.6
59	France		2003	red	nd
63	Italy	Primitivo di Manduria	2007	red	1.2
64	Italy		2005	red	nd
65	France		2007	red	nd
66	Italy	Corvina, Rondinello, Molinara	2004	red	3.2
67	Spain	Monastrell	2008	red	nd
68	France	Grenache, Syrah, Mourvedre	2008	red	6.5
71	France	Merlot, Cabernet Sauvignon, Cabernet Franc	2003	red	nd
72	France		2002	red	nd
73	Portugal			port	nd
74	France			white	nd
78	Italy	Cabernet Sauvignon, Cabernet Franc	2001	red	nd
79	France	Riesling	2008	white	nd
80	Spain		2004	red	nd
84	France	Cabernet Sauvignon	1996	red	nd
89	Italy		2008	white	1.0
90	France		2007	red	nd
91	Italy	Sangiovese, Rondinella, Corvina	2006	red	nd
92	Spain	Tempranillo	2005	red	nd
93	France		1991	red	nd
94	France		1996	red	nd
95	Italy			red	nd
96	Chile	Carmenere	2008	red	nd

Table 1. Continued

sample number	country	grape sort	year	wine type	fumonisin (μg/L)
99	Argentina	Cabernet, Merlot	2008	red	nd
100	Italy			rose	nd
101	Spain		1996	red	nd
102	France		1998	red	nd
103	Portugal			port	nd
104	Portugal			port	2.8
105	Portugal			port	nd
106	Spain		2003	red	nd
107	Portugal		1991	red	nd
108	France	Cabernet Sauvignon, Merlot Noir, Cabernet Franc	1994	red	nd

[a] The description of the wines was read off the label of the bottle. Blank cells indicate that information was not available. [b] nd = not detected.

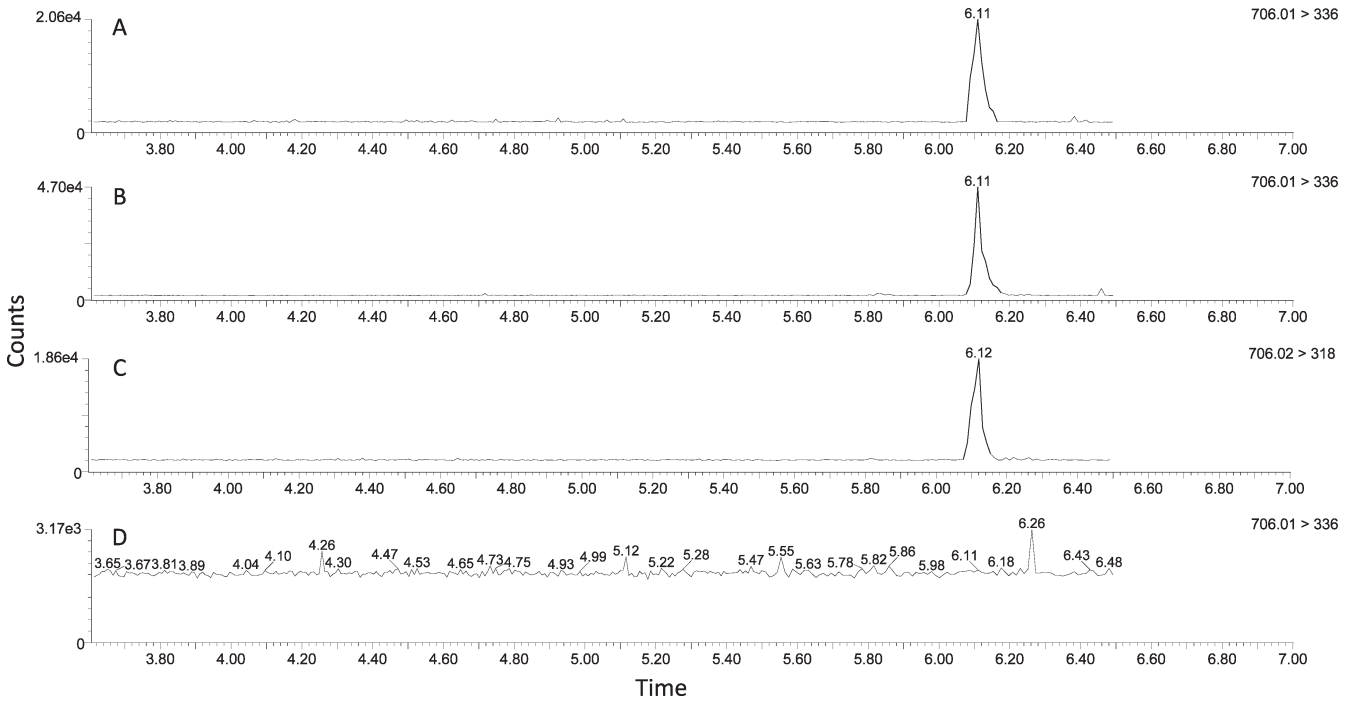

Figure 2. Results from the LC−MS/MS analysis. Transitions of fumonisin B_2 in spiked wine and a wine sample. (A) Quantifier of fumonisin B_2 from spiked wine (45 μg/L). (B) Quantifier of fumonisin B_2 from a wine sample (25 μg/L). (C) Qualifier of fumonisin B_2 from a wine sample (25 μg/L). (D) Quantifier of fumonisin B_2 from a blank sample (0 μg/L).

the ^{13}C fumonisin B_2, the recovery seemed to be better than 90%. However, clearly, it should not be necessary to use immunoaffinity purification in combination with LC−MS/MS because of the selectivity of the latter technique. The extra purification of 24 samples included 5 control samples (1 blank and 4 spiked with 2−45 μg/L). Re-analysis of the 18 initial positive samples did not change the number of positive samples, further confirming that the findings were true positive results. Of the 18 positive samples, 16 were red wine, 1 was white wine, and 1 was port wine (**Table 1**). The positive samples contained 1−25 μg/L fumonisin B_2 (average, 5.2 μg/L; median, 2.6 μg/L). Selected tested samples are shown in **Figure 2**. On the basis of the relative low amounts of samples, it is indicated that the fumonisin contamination could pose a larger problem in red wine (28% positive) compared to white wine (7% positive). This is similar to the ochratoxin A contamination, which is found to be at a higher concentration and coincidence in red wine compared to white wine (*18*, *19*). The findings of fumonisin B_2 with such a high frequency show that *A. niger* is apparently commonly growing on grapes in the fields. However, the frequency of heavily infected berries was low because the detected levels are 100−400 lower than those found in highly infected berries (2−8 mg/L) (*10*, *11*). Levels of ochratoxin A in wine have been found to be at 0.002−7.63 μg/L (*19*, *20*), which are lower than the found fumonisin concentrations. There were no statistically significant differences between the fumonisin concentration and countries and years, because of the relatively few samples investigated here.

The maximum permitted level of ochratoxin A in wine is set at 2.0 μg/L by the European Community (EC); this is ca. 2−3 times lower than the average maximum permitted level of ochratoxin A in food (*21*, *22*). Thus, a crude comparison to fumonisin, one could expect a 2−3-fold lower level than the 1−2 mg/kg set by the EC (*14*) and U.S. FDA (*13*), respectively. Thus, the maximum level detected (25 μg/L) was 40−80 times lower than an anticipated regulatory limit set by the EC and U.S. FDA.

However, even though the detected fumonisin concentrations are below such a limit, it is necessary to perform a larger survey to establish if wines with even higher concentrations are produced, because climatic and production conditions vary. Exposure assessment needs to be performed, especially concerning groups

with a high intake of wine alone or combined with other fumonisin-contaminated products.

In conclusion, this is the first report on the detection of fumonisin B_2 in wine, showing that almost a quarter of the samples were positive. This clearly points toward a much larger survey of how widespread fumonisins are in wine, as well as a determination of the fate of fumonisins in the winemaking process.

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LITERATURE CITED

(1) Pitt, J. I.; Hocking, A. D. *Fungi and Food Spoilage*, 2nd ed.; Blackie Academic and Professional: London, U.K., 1997.
(2) Varga, J.; Juhasz, A.; Kevei, F.; Kozakiewicz, Z. Molecular diversity of agriculturally important *Aspergillus* species. *Eur. J. Plant Pathol.* **2004**, *110*, 627–640.
(3) Abarca, M. L.; Accensi, F.; Bragulat, M. R.; Castella, G.; Cabañes, F. J. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. *J. Food Prot.* **2003**, *66*, 504–506.
(4) Battilani, P.; Giorni, P.; Bertuzzi, T.; Formenti, S.; Pietri, A. Black aspergilli and ochratoxin A in grapes in Italy. *Int. J. Food Microbiol.* **2006**, *111*, S53–S60.
(5) Belli, N.; Pardo, E.; Marin, S.; Farre, G.; Ramos, A. J.; Sanchis, V. Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. *J. Sci. Food Agric.* **2004**, *84*, 541–546.
(6) Cabañes, F. J.; Accensi, F.; Bragulat, M. R.; Abarca, M. L.; Castella, G.; Minguez, S.; Pons, A. What is the source of ochratoxin A in wine? *Int. J. Food Microbiol.* **2002**, *79*, 213–215.
(7) Frisvad, J. C.; Smedsgaard, J.; Samson, R. A.; Larsen, T. O.; Thrane, U. Fumonisin B_2 production by *Aspergillus niger*. *J. Agric. Food Chem.* **2007**, *55*, 9727–9732.
(8) Noonim, P.; Mahakarnchanakul, W.; Nielsen, K. F.; Frisvad, J. C.; Samson, R. A. Fumonisin B_2 production by *Aspergillus niger* from Thai coffee beans. *Food Addit. Contam.* **2009**, *26*, 94–100.
(9) Månsson, M.; Klejnstrup, M. L.; Phipps, R. K.; Nielsen, K. F.; Frisvad, J. C.; Gotfredsen, C. H.; Larsen, T. O. Isolation and NMR characterization of fumonisin B_2 and a new fumonisin B_6 from *Aspergillus niger*. *J. Agric. Food Chem.* **2010**, *58*, 949–953.
(10) Logrieco, A.; Ferracane, R.; Haidukowsky, M.; Cozzi, G.; Visconti, A.; Ritieni, A. Fumonisin B_2 production by *Aspergillus niger* from grapes and natural occurrence in must. *Food Addit. Contam., Part A* **2009**, *26*, 1495–1500.
(11) Mogensen, J. M.; Frisvad, J. C.; Thrane, U.; Nielsen, K. F. Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. *J. Agric. Food Chem.* **2010**, *58*, 954–958.
(12) Sydenham, E. W.; Shephard, G. S.; Thiel, P. G.; Marasas, W. F. O.; Stockenstrom, S. Fumonisin contamination of commercial corn-based human foodstuffs. *J. Agric. Food Chem.* **1991**, *39*, 2014–2018.
(13) United States Food and Drug Administration (U.S. FDA). U.S. Food and Drug Administration Guidance for Industry: Fumonisin levels in human foods and animal feeds. U.S. FDA, Washington, D. C., **2001**.
(14) European Economic Community (EEC). Setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. *Comm. Eur. Communities* **2007**, *1126*, 14–16.
(15) Krska, R.; Welzig, E.; Boudra, H. Analysis of *Fusarium* toxins in feed. *Anim. Feed Sci. Technol.* **2007**, *137*, 241–264.
(16) Shephard, G. S. Determination of mycotoxins in human foods. *Chem. Soc. Rev.* **2008**, *37*, 2468–2477.
(17) European Economic Community (EEC). The performance of analytical methods and interpretations of results. *Comm. Eur. Communities* **2002**, *657*, 8–36.
(18) Otteneder, H.; Majerus, P. Occurrence of ochratoxin A (OTA) in wines: Influence of the type of wine and its geographical origin. *Food Addit. Contam.* **2000**, *17*, 793–798.
(19) Battilani, P.; Pietri, A. Ochratoxin A in grapes and wine. *Eur. J. Plant Pathol.* **2002**, *108*, 639–643.
(20) Blesa, J.; Soriano, J. M.; Molto, J. C.; Manes, J. Factors affecting the presence of ochratoxin A in wines. *Crit. Rev. Food Sci.* **2006**, *46*, 473–478.
(21) European Economic Community (EEC). EU rules on ochratoxin A extended to coffee, wine and grape juice. European Comission Press Release, **2004**; IP/04/1215.
(22) European Economic Community (EEC). Setting maximum levels for certain contaminants in foodstuffs. *Comm. Eur. Communities* **2002**, *472*, 18–20.

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Paper 4

”Occurrence of fumonisins B_2 and B_4 in retail raisins”

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ARTICLE

Occurrence of Fumonisins B_2 and B_4 in Retail Raisins

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Concerns that raisins may be contaminated by fumonisins stem from the persistent occurrence of *Aspergillus niger* spores on raisins and the recent discovery of fumonisin production by *A. niger* on grapes, which leads to the widespread occurrence of fumonisin B_2 in wine. This study presents an LC-MS/MS survey of fumonisins in retail raisins. In 10 of 21 brands collected in Denmark, Germany, and The Netherlands, fumonisins B_2 and B_4 were detected at levels up to 13 and 1.3 μg/kg, respectively. Only fumonisin B_2 has been detected in wine, so the presence of fumonisin B_4 in raisins suggests that the fumonisins are produced mainly during the drying process concomitant with the decreasing water activity. Analysis of multiple packages from one manufacturer showed a 3-fold package-to-package variation, suggesting that a few raisins per package are contaminated.

KEYWORDS: Fumonisins B_2 and B_4; wine; grapes; cation exchange; SPE; solid-phase extraction

INTRODUCTION

Black aspergilli are distributed globally and occur on a great variety of substrates. They are also widely used in the food and biotechnology industries for the production of organic acids, enzymes, and other products (*1*).

Black aspergilli are commonly found on the surface of healthy grapes during all growth stages (*2*). *Aspergillus* species are present on grape clusters early in the season, and their frequency increases in the later growth stages of the grapes (*3*). Of the various *Aspergillus* species, *Aspergillus niger* is by far the most commonly found on grapes and is shown in one study to occur on >80% of samples (*4*). Although *A. niger* is the predominant species, *Aspergillus carbonarius* is the most problematic because it consistently produces high amounts of ochratoxin A, whereas only 8−40% of *A. niger* strains produce low amounts of this toxin (*5*,*6*). This is the main mycotoxin-related health concern in grape-derived products. Nonetheless, *A. niger* production of fumonisins B_2, B_4, and B_6 (*7*−*9*) (**Figure 1**) and the high frequency with which fumonisin B_2 occurs in wine (*10*, *11*) raise concerns about the possible widespread presence of fumonisins in raisins. These concerns are further fuelled by the production of fumonisins B_2 and B_4 on artificially infected grapes and raisins to levels as high as 8 mg/kg (*12*,*13*), as well as a study that found *A. niger* spores on all raisins investigated (*13*). A recent analysis of seven raisin samples from around the world claimed fumonisin levels as high as 35 mg/kg, mainly for fumonisin B_2, but also detected fumonisins B_1, B_3, and B_6, as well as a number of tentatively identified fumonisin B_1 and B_2 OH-positional isomers (*14*). The finding of fumonisins B_1 and B_3 is unusual, because the *FUM2* gene encoding the enzyme for a C-10 hydroxylation that is required for the production of these isomers has not been detected in any of the three available full-genome sequences for *A. niger* strains (*15*, *16*). Furthermore, no other studies on fumonisin production in *A. niger* have detected fumonisin B_1 (*7*−*10*, *12*, *13*, *16*−*18*).

Structurally, fumonisins are similar to sphingolipids and have been shown to inhibit sphingolipid biosynthesis via the ceramide synthase pathway (*19*). The consumption of contaminated maize-based foods is associated with a high incidence of esophageal and liver cancers, along with neural tube defects (*20*). Furthermore, outbreaks of leukoencephalomalacia in horses and pulmonary edema and hydrothorax in pigs have been linked to consumption of fumonisin-contaminated feed (*20*, *21*). The regulatory limit for fumonisins in maize is 2−4 μg/g total fumonisins (*22*) in the United States and 0.2−2 μg/g in the European Union (*23*). Thus, the findings of 1−36 mg/kg in selected highly infected single raisins and artificially contaminated raisins clearly exceeds regulatory limits (*13*, *14*) and are therefore of public health concern. However, to our knowledge, no studies have reported average fumonisin levels in commercial raisins.

Raisin production methods vary in different parts of the world, but mainly involve sun-drying, shade-drying, or mechanical drying (*24*). Production of dark-colored raisins in the United States, Chile, and South Africa is by direct sun-drying (*24*), which usually takes between 2 and 4 weeks to reach the commercial limit of >85% dry matter (*24*, *25*). Faster drying may be obtained by the use of dipping solutions, commonly used in Mediterranean regions, where grapes are pretreated with solutions of 5−6% K_2CO_3 and olive oil to remove the waxy outer layer prior to sun-drying (*26*). This practice is generally used to produce the more light-colored sultana raisin variant (*24*−*26*). Golden variants are generally mechanically dried after pretreatment with SO_2 to retard nonenzymatic and enzymatic browning (*24*). Damaged grapes will likely become contaminated, especially by black aspergilli, during the drying period, because these fungi are present on both the grapes and the finished raisins (*13*). To keep

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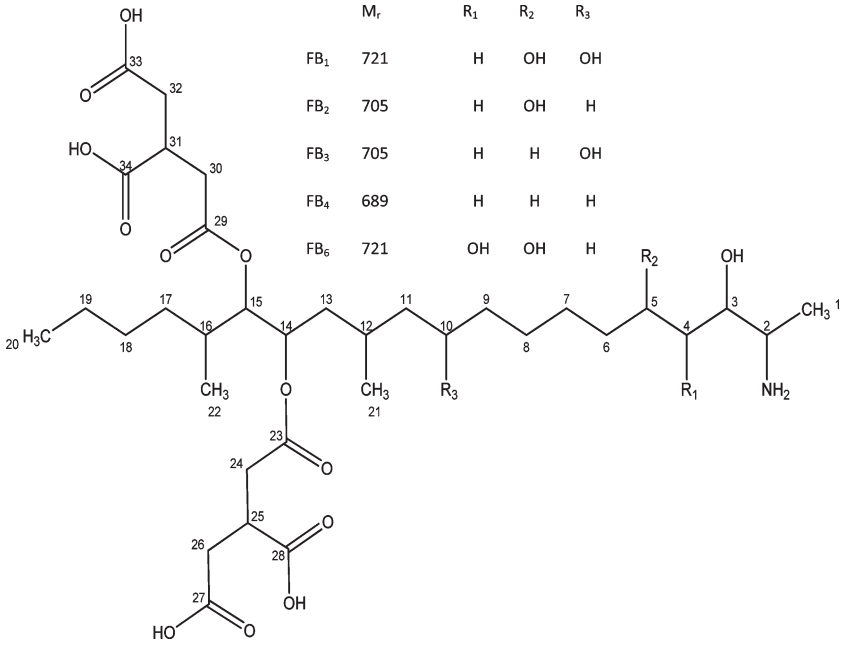

Figure 1. Structure of fumonisins B_1−B_4 and B_6.

ochratoxin A levels low, careful removal of infected raisins is enforced.

In this study, we investigated the presence of fumonisins in commercial raisins using cation-exchange purification with subsequent LC-MS/MS determination. We also investigated various extraction techniques to accommodate the high amounts of interfering material in raisins.

MATERIALS AND METHODS

All solvents were of HPLC grade, and other chemicals were of analytical grade. Reagents were from Sigma-Aldrich (St. Louis, MO). Water was purified on a Milli-Q system (Millipore, Bedford, MA). The fumonisin standard was a mixed certified standard containing fumonisins B_1 at 50.2 mg/L and B_2 at 51.0 mg/L (Biopure, Tulln, Austria). Fumonisin B_3 was from Biopure, and fumonisins B_4 and B_6 were from previous studies (*8*).

Raisin Samples. Raisins of 21 different brands were purchased from supermarkets and specialty shops in Denmark, Germany, and The Netherlands. The raisins were from the United States/California ($n = 9$), Greece ($n = 1$), South Africa ($n = 2$), Chile ($n = 5$), Turkey ($n = 3$), and China ($n = 1$). The 21 different brands represented a total of six different grape varieties including Thompson seedless, Jumbo Regal seedless, Flame, Sultana, Zante Currant, and Golden seedless.

Optimization of Sample Preparation. Raisins were homogenized with water (1:1 w/w) in a domestic kitchen blender. The mash was subsequently spiked with fumonisins and extracted. For method development, mixtures of water/methanol, water/ethanol, and water/acetonitrile, in 10% stepwise concentration increases from 30 to 100% organic solvent, were tested and the extracts purified using several formats (30 or 60 mg in 1 or 3 mL) of Strata X-C columns (Phenomenex, Torrance, CA). The effects of formic acid addition during extraction and different extraction volumes (3−20 times the amount of mash) were tested, along with different concentrations of NH_4OH for elution from the cation-exchange columns.

Sample Preparation. Raisins (≥200 g) were homogenized as described above, and 34 g of mash was transferred and split into two 50 mL Falcon tubes. The homogenates in each tube were extracted with 40 mL of acetonitrile/water/formic acid (86:12:2 v/v/v) under continuous shaking for 2 h. Samples were then centrifuged at 8000*g* for 5 min, and 35 mL of the upper phase of the resulting two-phase system was transferred to a clean 50 mL conical tube. Samples were evaporated to dryness under a N_2 flow, and each was redissolved in 10 mL of 1:1 v/v methanol/water, passed through a 0.45 μm PTFE syringe filter, and pooled. Sample purification was carried out using 60 mg/3 mL Strata X-C columns that had been preconditioned with 1 mL of methanol and 1 mL of water prior to sample load. To further acidify the column, 1 mL of water containing 2% formic acid was added, with a subsequent wash with 1 mL of methanol. Fumonisins were eluted with 1 mL of methanol/2% NH_4OH (1:4 v/v) into an autosampler vial and used directly for analysis.

LC-MS/MS conditions. LC-MS/MS analysis was performed as previously described (*11*, *13*) on a Quattro Ultima triple mass spectrometer (Micromass, Manchester, U.K.) using positive electrospray ionization and operated in multiple-reaction mode (MRM or SRM) in the following transitions: fumonisins B_2 and B_3, quantifier m/z 706 → 336, cone, 50 V; collision, 40 eV; dwell time, 50 ms; qualifier m/z 706 → 318; cone 50 V; collision, 25 eV; dwell time, 100 ms; fumonisin B_4, quantifier m/z 690 → 320; cone, 50 V; collision, 35 eV; dwell time, 50 ms; qualifier m/z 690 → 338; cone, 50 V; collision, 30 eV; dwell time, 100 ms; fumonisins B_1 and B_6, quantifier m/z 722 → 334; cone, 50 V; collision, 40 eV; dwell time, 50 ms; qualifier m/z 722 → 528; cone, 50 V; collision, 25 eV; dwell time, 100 ms.

Calibration. Quantification was by external standard quantification using linear regression, with comparison to spiked raisin samples (raisins/water 1:1 w/w) incubated for 2 h prior to extraction. Samples were spiked with a certified mixture of fumonisins B_1 and B_2 to 0 (blank), 1, 5, 15, 50, or 100 μg/kg dry raisins. Preliminary results had indicated that the samples had low fumonisin levels that were close to the limit of quantitation (LOQ), so each homogenized sample was analyzed in triplicate. Because available amounts of fumonisins B_4 were low, its response factor relative to fumonisin B_2 was determined on the basis of pure standards.

Validation. Fumonisin B_2 determination was validated by spiking portions of 34 g of raisin mash (raisin/water 1:1 w/w) with a mixture of fumonisins B_1 and B_2 to concentrations of 0 (blank), 1, 5, 15, 50, or 100 μg/kg dry raisins. Experiments were performed on three different days, with all levels assayed in triplicate each day. Apparent recovery was calculated as the ratio of the slope of the spiked raisins to a standard dilution series in methanol/water (20:80 v/v) diluted to 0, 1, 5, 15, 50, and 100 μg/kg. Fumonisins B_6 and B_3 were occasionally coanalyzed in sequence to ensure sufficient separation.

RESULTS AND DISCUSSION

Sample Purification. Fumonisins have traditionally been purified using anion-exchange or immunoaffinity columns; however, in previous studies we observed that raisin extracts fouled immunoaffinity columns, probably due to polysaccharides and brown pigments (*13*), resulting in brownish viscous methanol eluates. Poor recovery has been observed when using anion-exchange columns for grape extracts and wine, most likely

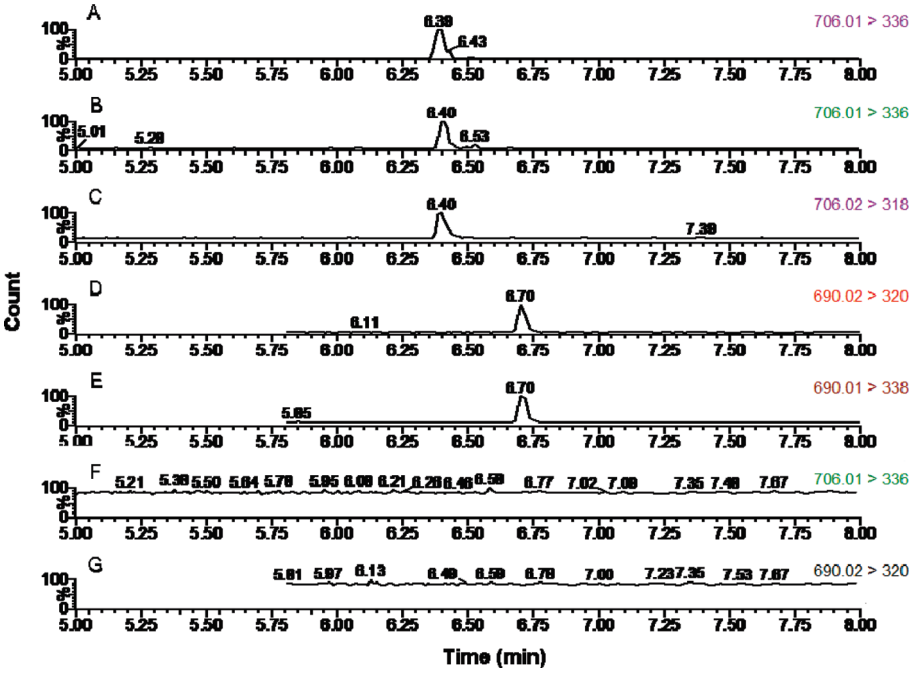

Figure 2. LC-MS/MS analysis; fumonisin B_2 and B_4 transitions in a fumonisin B_2-spiked sample and a blank raisin sample: (**A**) quantifier of fumonisin B_2 in a spiked raisin sample (5 μg/kg); (**B**) quantifier and (**C**) qualifier of fumonisin B_2 in a raisin sample (4.6 μg/kg); (**D**) quantifier and (**E**) qualifier of fumonisin B_4 in a raisin sample (1.2 μg/kg); (**F**) quantifier of fumonisin B_2 in a noncontaminated raisin sample; (**G**) quantifier of fumonisin B_4 in a noncontaminated sample.

because of the high content of organic acids, which salt-out fumonisins (*11*, *13*). To circumvent this problem, we used polymeric mixed-mode cation reversed phase columns, which target the amine of the fumonisins and have been proven effective for purifying fumonisins from wine (*11*).

During method development, we tested ethanol/water and methanol/water mixtures for extraction, because they did not result in a two-phase system as acetonitrile/water did, as also observed for aflatoxin extraction in various dry sample types (*27*). However, extensive amounts of pigments and polysaccharides (as judged from the extract viscosity) subsequently interfered with solid-phase extraction column function. Because this did not occur using acetonitrile extracts, we selected this QuEChERS-like method (*28*) in which the acetonitrile/water phase separates because of sugars from the raisins and performed further optimizations on this. Not surprisingly, the extracts needed to be acidified to keep the fumonisins in the acetonitrile phase. We also found that the volume of acetonitrile increased with decreasing temperature (observed when extracted stored in the refrigerator), so the samples were processed at the same temperature as the spiked samples used for calibration. We used two 50 mL conical tubes instead of a single 100 mL tube for extraction and extract evaporation to fit our centrifuge and N_2 evaporation equipment.

Validation. The LOQ was determined to be 1 μg/kg for fumonisin B_2 at a relative standard deviation of 35% ($n = 9$), which is highly satisfactory for this low level in this type of matrix. Trueness was 89, 101, and 97% for the three days, with average deviations of 22, 8, and 9%. The limit of detection (LOD, s/n 5) was at least 0.3 μg/kg for fumonisin B_2 and 0.3 and 0.1 μg/kg for fumonisin B_4. Apparent recovery was 87 ± 15%.

Screening of Raisin Samples. Our analysis demonstrated that 48% of the 21 tested raisin brands contained fumonisin B_2 and 43% fumonisin B_4. The detected amounts of fumonisins B_2 and B_4 in the positive samples ranged from the LOD (0.3 and 0.1 μg/kg) to 13 and 1.3 μg/kg, respectively. LC-MS/MS MRM chromatograms from selected spiked and nonspiked samples are shown in **Figure 2**.

The detection of fumonisin B_4 from retail raisins matches previous studies on artificially infected grapes and raisins (*13*); however, fumonisin B_4 was not identified in studies of fumonisin content in wine and grape must (*10*−*12*). This might suggest a different growth physiology and metabolism of *A. niger* on the drying grapes, possibly because of a fast fungal growth rate that results in some fumonisin B_4 not being transformed into B_2 before the water activity decreases below *A. niger* growth limits (~0.85) (*4*). This postharvest growth along with the physical removal of water from the vine fruit and the concomitant concentration of the fumonisins may explain why fumonisin B_4 has not been detected in previous wine studies (*10*, *11*). The presence of fumonisin B_4 in raisins could also explain our higher frequency of positive samples because, in contrast to wine production, both the postharvest period and the drying are sources of fumonisin production and upconcentration.

The frequency of fumonisin contamination in wine is approximately 18−23% (*10*, *11*), which is half the frequency observed in the raisins in this study, fitting well with the higher frequency being caused by postharvest growth of *A. niger*. This correlates well with studies demonstrating a significant increase in fungal load during drying, exceeding the expected increase due to the concentration effect of water evaporation(*4*).

The levels we detected were > 1000-fold lower than in samples analyzed by Varga et al. (*14*), in which fumonisin B_1 was also detected. The finding of fumonisin B_1 is highly unlikely because bioinformatic analysis of the data from the three fully sequenced *A. niger* strain genomes (*15*) has not detected a *FUM2* gene encoding the enzyme responsible for C-10 hydroxylation (*29*). Thus, *A. niger* should not produce fumonisins B_1 and B_3 as also reported here and in all other studies analyzing extracts from *A. niger* strains (*7*−*10*, *12*, *13*, *16*−*18*).

Of the 10 positive samples in this study, 7 were derived from California ($n = 9$), 2 from Chile, and 1 from Turkey (**Table 1**), demonstrating a higher infection rate in the California raisins. The reasons for this are unknown and could be because of the different climate, preharvest weather conditions, different grape

Table 1. Overview of Tested Raisin Samples and Average Fumonisin Content[a]

brand	origin	grape variety	organic	fumonisin B_2 (μg/kg)	fumonisin B_4[b] (μg/kg)
1	California	Thompsons Seedless		4.9 ± 0.4	0.26 ± 0.05
1				4.5 ± 1	0.8 ± 0.4
1				13.0 ± 1	1.3 ± 0.4
1				7.5 ± 0.8	0.9 ± 0.2
2	California	Thompsons Seedless		2.7 ± 0.2	0.29 ± 0.1
3	California	Thompsons Seedless	√	1.8 ± 0.2	<LOQ[c]
4	California	Thompsons Seedless	√	2.6 ± 0.4	0.83 ± 0.5
5	California	Thompsons Seedless		3.8 ± 0.6	0.31 ± 0.09
6	California	Thompsons Seedless		4.1 ± 0.6	1.0 ± 0.1
7	California	Thompsons Seedless	√	1.3 ± 0.6	0.25 ± 0.2
8	California	Thompsons Seedless		ND[d]	ND
9	California	Thompsons Seedless	√	ND	ND
10	Turkey	Sultana	√	ND	ND
11	Turkey	Sultana	√	ND	ND
12	Turkey	Sultana		4.8 ± 2	0.75 ± 0.1
13	Greece	Zante currants		ND	ND
14	South Africa	Golden Seedless		ND	ND
15	South Africa	Jumbo Regal Seedless	√	ND	ND
16	China	unknown		ND	ND
17	Chile	Flame		ND	ND
18	Chile	Flame		ND	ND
19	Chile	Flame		ND	ND
20	Chile	Golden		<LOQ	ND
21	Chile	Golden		1.4 ± 0.6	<LOQ

[a] Fumonisin concentration is the average of three determinations from the same package, ±one standard deviation. [b] Fumonisin B_4 was calculated assuming a 4-fold better response factor than for fumonisin B_2 and is shown from one spiking experiment. Fumonisins B_1 and B_6 were not detected in any of the packages. [c] LOQ, limit of quantification: for fumonisin B_2, 1 μg/kg; for fumonisin B_4, estimated as 0.25 μg/kg. [d] ND, not detected. The limit of detection for fumonisin B_2 was 0.3 μg/kg, and that for fumonisin B_4 was 0.1 μg/kg.

cultivars, or different drying and sorting techniques (*4*). No connection was observed between fumonisin content and type of cultivation strategy (organic/conventional).

Large package variations were observed with up to 3-fold differences between four packages of the same brand, indicating a nonhomogeneous infection level, which may be due to contaminated raisins. Even though the detected fumonisin levels were 50−100 times below the regulatory limits of similar commodities such as maize, a larger survey is needed, both because of the low number of retail brands tested and because of the high variation among packages. Furthermore, establishing whether raisins may have even higher concentrations under different climatic conditions and drying procedures is important.

We presume that the low levels that we report here are also because of the efficient removal of damaged grapes that was initiated after problems with ochratoxin A in grapes and derived products were reported in the late 1990s (*30*−*32*), leading to very strict European Community regulations (472/2002) (*33*), including a maximum allowance of 10 μg/kg ochratoxin A in dried vine fruits (*34*).

In conclusion, this first survey on fumonisin determination in retail raisins demonstrated that almost 50% of tested brands contained low levels of fumonisins (1−14 μg/kg), which is far from the maximum allowable levels in regulated foods such as maize. Nonetheless, our finding of a high frequency of fumonisin occurrence clearly calls for larger surveys that can establish whether certain regions or weather conditions lead to fumonisin concentrations above set regulatory limits, as well as investigations of the influence of the drying process, especially on the production of fumonisin B_4.

LITERATURE CITED

(1) Schuster, E.; Dunn-Coleman, N.; Frisvad, J. C.; van Dijck, P. W. M. On the safety of *Aspergillus niger* − a review. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 426−435.

(2) Zahavi, T.; Cohen, L.; Weiss, B.; Schena, L.; Daus, A.; Kaplunov, T.; Zutkhi, J.; Ben-Arie, R.; Droby, S. Biological control of *Botrytis*, *Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. *Postharvest Biol. Technol.* **2000**, *20*, 115−124.

(3) Battilani, P.; Giorni, P.; Pietri, A. Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape. *Eur. J. Plant Pathol.* **2003**, *109*, 715−722.

(4) Leong, S. L.; Hocking, A. D.; Pitt, J. I. Occurrence of fruit rot fungi (*Aspergillus* section Nigri) on some drying varieties of irrigated grapes. *Aust. J. Grape Wine Res.* **2004**, *10*, 83−88.

(5) Magnoli, C.; Violante, M.; Combina, M.; Palacio, G.; Dalcero, A. Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. *Lett. Appl. Microbiol.* **2003**, *37*, 179−184.

(6) Da Rocha Rosa, C. A. D.; Palacios, V.; Combina, M.; Fraga, M. E.; Rekson, A. D.; Magnoli, C. E.; Dalcero, A. M. Potential ochratoxin A producers from wine grapes in Argentina and Brazil. *Food Addit. Contam.* **2002**, *19*, 408−414.

(7) Frisvad, J. C.; Smedsgaard, J.; Samson, R. A.; Larsen, T. O.; Thrane, U. Fumonisin B_2 production by *Aspergillus niger*. *J. Agric. Food Chem.* **2007**, *55*, 9727−9732.

(8) Månsson, M.; Klejnstrup, M. L.; Phipps, R. K.; Nielsen, K. F.; Frisvad, J. C.; Gotfredsen, C. H.; Larsen, T. O. Isolation and NMR characterization of fumonisin B_2 and a new fumonisin B_6 from *Aspergillus niger*. *J. Agric. Food Chem.* **2010**, *58*, 949−953.

(9) Noonim, P.; Mahakarnchanakul, W.; Nielsen, K. F.; Frisvad, J. C.; Samson, R. A. Fumonisin B_2 production by *Aspergillus* niger in Thai coffee beans. *Food Addit. Contam. Part A: Chem. Anal. Control Expo. Risk Assess.* **2009**, *26*, 94−100.

(10) Logrieco, A.; Ferracane, R.; Visconti, A.; Ritieni, A. Natural occurrence of fumonisin B_2 in red wine from Italy. *Food Addit. Contam. Part A: Chem. Anal. Control Expo. Risk Assess.* **2010**, *27*, 1136−1141.

(11) Mogensen, J. M.; Larsen, T. O.; Nielsen, K. F. Widespread occurrence of the mycotoxin fumonisin B_2 in wine. *J. Agric. Food Chem.* **2010**, *58*, 4853−4857.

(12) Logrieco, A.; Ferracane, R.; Haidukowsky, M.; Cozzi, G.; Visconti, A.; Ritieni, A. Fumonisin B_2 production by *Aspergillus niger* from grapes and natural occurrence in must. *Food Addit. Contam. Part A: Chem. Anal. Control Expo. Risk Assess.* **2009**, *26*, 1495−1500.

(13) Mogensen, J. M.; Frisvad, J. C.; Thrane, U.; Nielsen, K. F. Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. *J. Agric. Food Chem.* **2010**, *58*, 954−958.

(14) Varga, J.; Kocsube, S.; Suri, K.; Szigeti, G.; Szekeres, A.; Varga, M.; Toth, B.; Bartok, T. Fumonisin contamination and fumonisin producing black aspergilli in dried vine fruits of different origin. *Int. J. Food Microbiol.* **2010**, *143*, 143−149.

(15) Pel, H. J.; de Winde, J. H.; Archer, D. B.; Dyer, P. S.; Hofmann, G.; Schaap, P. J.; Turner, G.; de Vries, R. P.; Albang, R.; Albermann, K.; Andersen, M. R.; Bendtsen, J. D.; Benen, J. A. E.; van den Berg, M.; Breestraat, S.; Caddick, M. X.; Contreras, R.; Cornell, M.; Coutinho, P. M.; Danchin, E. G. J.; Debets, A. J. M.; Dekker, P.; van Dijck, P. W. M.; van Dijk, A.; Dijkhuizen, L.; Driessen, A. J. M.; d'Enfert, C.; Geysens, S.; Goosen, C.; Groot, G. S. P.; de Groot, P. W. J.; Guillemette, T.; Henrissat, B.; Herweijer, M.; van den Hombergh, J. P. T. W.; van den Hondel, C. A. M. J.; van der Heijden, R. T. J. M.; van der Kaaij, R. M.; Klis, F. M.; Kools, H. J.; Kubicek, C. P.; van Kuyk, P. A.; Lauber, J.; Lu, X.; van der Maarel, M. J. E. C.; Meulenberg, R.; Menke, H.; Mortimer, M. A.; Nielsen, J.; Oliver, S. G.; Olsthoorn, M.; Pal, K.; van Peij, N. N. M. E.; Ram, A. F. J.; Rinas, U.; Roubos, J. A.; Sagt, C. M. J.; Schmoll, M.; Sun, J. B.; Ussery, D.; Varga, J.; Vervecken, W.; de Vondervoort, P. J. J. V.; Wedler, H.; Wosten, H. A. B.; Zeng, A. P.; van Ooyen, A. J. J.; Visser, J.; Stam, H. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.* **2007**, *25*, 221−231.

(16) Susca, A.; Proctor, R. H.; Mule, G.; Stea, G.; Ritieni, A.; Logrieco, A.; Moretti, A. Correlation of mycotoxin fumonisin B_2 production and presence of the fumonisin biosynthetic gene fum8 in *Aspergillus niger* from grape. *J. Agric. Food Chem.* **2010**, *58*, 9266−9272.

(17) Mogensen, J. M.; Nielsen, K. F.; Frisvad, J. C.; Thrane, U. Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* species. *BMC Microbiol.* **2009**, *9*, 281.

(18) Nielsen, K. F.; Mogensen, J. M.; Johansen, M.; Larsen, T. O.; Frisvad, J. C. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Anal. Bioanal. Chem.* **2009**, *395*, 1225−1242.

(19) Marasas, W. F.; Riley, R. T.; Hendricks, K. A.; Stevens, V. L.; Sadler, T. W.; Gelineau-van, W. J.; Missmer, S. A.; Cabrera, J.; Torres, O.; Gelderblom, W. C.; Allegood, J.; Martinez, C.; Maddox, J.; Miller, J. D.; Starr, L.; Sullards, M. C.; Roman, A. V.; Voss, K. A.; Wang, E.; Merrill, A. H., Jr. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. *J. Nutr.* **2004**, *134*, 711−716.

(20) Stockmann-Juvala, H.; Savolainen, K. A review of the toxic effects and mechanisms of action of fumonisin B1. *Hum. Exp. Toxicol.* **2008**, *27*, 799−809.

(21) Yazar, S.; Omurtag, G. Z. Fumonisins, trichothecenes and zearalenone in cereals. *Int. J. Mol. Sci.* **2008**, *9*, 2062−2090.

(22) U.S. Food and Drug Administration. *Guidance for Industry: Fumonisin Levels in Human Foods and Animal Feeds*; U.S. Food and Drug Administration: Washington, DC, 2001.

(23) EC 1126/2007 amending Regulation No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. *Commission Eur. Communities* **2007**, *1126*, 14−16.

(24) Esmaiili, M.; Sotudeh-Gharebagh, R.; Cronin, K.; Mousavi, M. A. E.; Rezazadeh, G. Grape drying: a review. *Food Rev. Int.* **2007**, *23*, 257−280.

(25) Dincer, I. Sun drying of Sultana grapes. *Dry. Technol.* **1996**, *14*, 1827−1838.

(26) Doymaz, I.; Pala, M. The effects of dipping pretreatments on air-drying rates of the seedless grapes. *J. Food Eng.* **2002**, *52*, 413−417.

(27) Stroka, J.; Petz, M.; Joerissen, U.; Anklam, E. Investigation of various extractants for the analysis of aflatoxin B-1 in different food and feed matrices. *Food Addit. Contam.* **1999**, *16*, 331−338.

(28) Anastassiades, M.; Lehotay, S. J.; Stajnbaher, D.; Schenck, F. J. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *J. AOAC Int.* **2003**, *86*, 412−431.

(29) Butchko, R. A. E.; Plattner, R. D.; Proctor, R. H. Deletion analysis of FUM genes involved in tricarballylic ester formation during fumonisin biosynthesis. *J. Agric. Food Chem.* **2006**, *54*, 9398−9404.

(30) Majerus, P.; Otteneder, H. Detection and occurrence of ochratoxin A in wine and grapejuice. *Dtsch. Lebensm.-Rundsch.* **1996**, *92*, 388−390.

(31) Zimmerli, B.; Dick, R. Ochratoxin A in table wine and grape-juice: occurrence and risk assessment. *Food Addit. Contam.* **1996**, *13*, 655−668.

(32) Burdaspal, P.; Legarda, T. Ochratoxin A in wines and grape products originated from Spain and other european countries. *Alimentaria* **1999**, *36*, 107−113.

(33) EC 1881/2006. Setting maximum levels for certain contaminants in foodstuffs. *Commission Eur. Communities* **2006**, *472*, 18−20.

(34) EC 472/2002. Setting maximum levels for certain contaminants in foodstuffs. *Commission Eur. Communities* **2002**, *472*, 18−20.

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Paper 5

" *Aspergillus acidus* from Puerh tea and black tea does not produce ochratoxin A and fumonisin B_2"

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Aspergillus acidus from Puerh tea and black tea does not produce ochratoxin A and fumonisin B_2

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ABSTRACT

Puerh tea is a unique Chinese fermented tea. Unlike other teas it is stored for a long period of time. *Aspergillus niger* is claimed to be the dominant microorganism in the Puerh tea manufacturing process and also to be common on tea in general. *A. niger sensu stricto* is known to produce the mycotoxins ochratoxin A, fumonisins B_2 and B_4. With this in mind, we performed a preliminary study to determine if production of these mycotoxins by black Aspergilli isolated from Puerh and black tea can occur. An examination of 47 isolates from Puerh tea and black tea showed that none of these was *A. niger*. A part of the calmodulin gene in 17 isolates were sequenced, and these 17 isolates were all identified as *Aspergillus acidus* (=*A. foetidus* var. *acidus*). The rest of the 47 isolates were also identified as *A. acidus* from their metabolite profile. Neither production of ochratoxin A nor fumonisins B_2 and B_4 by any of the 47 isolates were observed.

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1. Introduction

Puerh tea is a black tea, which is produced mainly in the Chinese Yunnan district and consumed by a large part of the Chinese population (Sano et al., 1986). The Puerh tea is different from other teas, in the sense that it is fermented as a normal black tea, but is matured for a period of time (Sano et al., 1986). It is said that the longer the maturation, the better taste and quality of the Puerh tea (Sano et al., 1986). According to the literature *Aspergillus niger* is associated with black tea and is the dominating microorganism during the Puerh tea production (Abe et al., 2008; Elshafie et al., 1999; Mo et al., 2005; Sano et al., 1986; Xu et al., 2005). *A. niger* and other members of section *Nigri* are difficult to classify and identify because they are morphologically very similar (Samson et al., 2007). Physiology, secondary metabolites and DNA sequencing can help to differentiate between the species (Samson et al., 2004; Samson et al., 2007).

Recently a putative fumonisin gene cluster was discovered in two genomes of *A. niger*, strains ATCC 1015 and CBS 513.88 (Baker 2006; Pel et al., 2007). This was followed by the discovery of the ability to produce fumonisins B_2 (FB_2) and B_4 (FB_4) by *A. niger* (Frisvad et al., 2007; Noonim et al., 2009). Besides this production *A. niger* is also a well known ochratoxin A (OTA) producer (Abarca et al., 1994). Fumonisin B_1 has previously been isolated from black tea (Martins et al., 2001), and since Puerh tea is presumably fermented with *A. niger*, there is a possibility that fumonisins and potentially OTA from *A. niger* can be found in Puerh tea. We therefore investigated the ability of 47 black Aspergilli, isolated from black tea and Puerh tea, to produce OTA as well as FB_2 and FB_4.

2. Materials and methods

Unless otherwise stated all solvents were HPLC grade, chemicals were analytical grade and water was purified on a Milli-Q system (Millipore, Bedford, MA). Media were prepared in 9 cm Petri dishes, each with 20 ml substrate, and strains were inoculated by the three point method and dishes incubated in micro perforated plastic bags for 7 days in darkness. All samples were as a minimum made in biological duplicates on two separate plates.

2.1. Samples

Ten different tea samples were purchased from the internet (www.tea.dk) or in supermarkets/specialty shops in Denmark or The Netherlands. All teas were end products, 5 were Puerh teas (Brand A–E) and 5 were regular black teas (Brand 1–5). The Puerh teas were all from the Yunnan Region, China. The black teas were from Anhui region (China), Singtom (India) or Sri Lanka.

2.2. Screening

One gram portions of each tea were taken at random from well mixed portions (min. 50 g). Afterwards, each portion was plated onto five Petri dishes, containing dichloran 18% glycerol agar (DG18) (Hocking and Pitt, 1980). The plates were incubated for seven days at 25 °C. After

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incubation, up to five black *Aspergillus* cultures from each tea were isolated and inoculated on Czapek yeast autolysate agar with 5% salt (CYAS) (Frisvad and Samson, 2004), malt extract agar (Pitt, 1979), yeast extract sucrose agar (YES) (Frisvad and Samson, 2004) and two Czapek yeast autolysate agar (CYA) (Frisvad and Samson, 2004). All plates were incubated for seven days at 25 °C. In addition a CYA plate incubated at 37 °C was used for each isolate. Morphological and physiological studies were performed using all five plates.

2.3. Analysis of secondary metabolites

Three plugs ($D=6$ mm) from the CYA (25 °C) and three plugs ($D=6$ mm) from the YES plate were transferred to a 2 ml dram vial and 800 µl of methanol:dichloromethane:ethyl acetate (1:2:3; vol:vol:vol) with 1% formic acid was added. The plugs were extracted ultrasonically for one hour. The extract was transferred to a new vial and the organic phase was evaporated in a rotational vacuum concentrator. The remains were redissolved by ultrasonication in 500 µl methanol for twenty minutes. The samples were filtrated through a FTPE 0.45 µm filter and then analyzed by HPLC-DAD (Smedsgaard et al., 1997). The potential FB_2 and FB_4 production were investigated by the extraction method described by Frisvad et al. in 2007 with small modifications. Extraction of two separate sets of six plugs from the CYAS and YES plate were performed. The plugs were extracted ultrasonically for one hour with 800 µl methanol:water (3:1). The extract was filtrated through a FTPE 0.45 µm filter and used directly for LC-MS.

2.4. HPLC conditions

Extracts were analyzed on a Agilent 1100 HPLC system equipped with a diode array detector. Scan: 190–600 nm with a bandwidth of 4 nm approx. two times per second. The separation was performed on a 100×2 mm, 3 µm particle size, i.d. Luna C-18 (II) column fitted with a 4×2 mm, 3 µm particle size, C18 security guard column. Using a water: acetonitrile, the gradient started at 15% acetonitrile and was increased to 100% acetonitrile in twenty minutes and kept there for five minutes, before it returned to starting conditions. The flow rate was 0.3 ml/min. Both eluents contained 0.005% (v/v) trifluoroacetic acid (modified from Smedsgaard et al., 1997). All chemicals were analytical grade. 3 µl extract was injected for all samples.

Table 1
Known metabolites produced by 47 black Aspergilli isolated from tea.

IBT no.	Type of tea (Brand)	Asperazine	Funalenone	Pyranonigin A	Naphto-γ-pyrones	Antafumicin	FB_2	FB_4	OTA	Identified as
24798	Black tea (1)	X	X	X	X	X	–	–	–	*A. acidus*
24799	Black tea (1)	X	X	X	X	X	–	–	–	*A. acidus*
24800	Black tea (1)	X	X	X	X	X	–	–	–	*A. acidus*
24801	Black tea (2)	X	X	X	X	X	–	–	–	*A. acidus*
24802	Black tea (2)	X	X	X	X	X	–	–	–	*A. acidus*
24803	Black tea (2)	X	X	X	X	X	–	–	–	*A. acidus*
24821	Black tea (3)	X	X	X	X	X	–	–	–	*A. acidus*
24822	Black tea (3)	X	X	X	X	X	–	–	–	*A. acidus*
24825	Black tea (3)	X	X	X	X	X	–	–	–	*A. acidus*
24826	Black tea (3)	X	X	X	X	X	–	–	–	*A. acidus*
24830	Black tea (3)	X	X	–	X	X	–	–	–	*A. acidus*
24831	Black tea (3)	X	X	X	X	X	–	–	–	*A. acidus*
29563	Black tea (4)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29564	Black tea (4)	X	X	–	X	–	–	–	–	*A. acidus*
29565	Black tea (4)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29566	Black tea (4)	X	X	–	X	–	–	–	–	*A. acidus*
29567	Black tea (4)	X	X	X	X	–	–	–	–	*A. acidus*
29568	Puerh tea (A)	X	X	–	X	–	–	–	–	*A. acidus*
29569	Puerh tea (A)	X	X	X	X	–	–	–	–	*A. acidus*
29570	Puerh tea (A)	X	X	–	X	–	–	–	–	*A. acidus*[a]
29571	Puerh tea (A)	X	X	–	X	–	–	–	–	*A. acidus*[a]
29572	Puerh tea (B)	X	X	X	–	–	–	–	–	*A. acidus*
29573	Puerh tea (B)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29574	Puerh tea (B)	X	X	X	X	–	–	–	–	*A. acidus*
29575	Puerh tea (B)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29576	Puerh tea (B)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29577	Puerh tea (C)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29578	Puerh tea (C)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29579	Puerh tea (C)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29580	Puerh tea (C)	X	X	X	X	–	–	–	–	*A. acidus*
29581	Puerh tea (C)	X	X	–	X	–	–	–	–	*A. acidus*
29582	Puerh tea (D)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29583	Puerh tea (D)	X	X	X	X	–	–	–	–	*A. acidus*
29584	Puerh tea (D)	X	X	X	X	–	–	–	–	*A. acidus*
29585	Puerh tea (D)	X	X	X	–	–	–	–	–	*A. acidus*[a]
29586	Puerh tea (D)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29587	Black tea (5)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29588	Black tea (5)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29589	Black tea (5)	X	X	X	X	–	–	–	–	*A. acidus*
29590	Black tea (5)	X	X	–	X	–	–	–	–	*A. acidus*
29591	Black tea (5)	X	X	X	X	–	–	–	–	*A. acidus*
29592	Puerh tea (E)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29593	Puerh tea (E)	X	X	X	X	–	–	–	–	*A. acidus*
29594	Puerh tea (E)	X	X	X	X	–	–	–	–	*A. acidus*
29595	Puerh tea (E)	X	X	–	–	–	–	–	–	*A. acidus*
29596	Puerh tea (E)	X	X	X	X	–	–	–	–	*A. acidus*[a]
29597	Puerh tea (A)	X	X	X	X	–	–	–	–	*A. acidus*

[a] Identified from molecular data.

2.5. LC-MS conditions

The LC-MS analysis was performed on a LC/MSD VL single quadrupole (Agilent, Santa Clara, California, USA). The separation was done on a 50×2 mm, i.d. 3 µm particle size, Luna C-18 (II) column (Phenomenex, Torrance, California), which was heated to 40 °C, fitted with a security guard column, using a water:acetonitrile (both containing 20 mM formic acid) gradient at a flow rate of 0.3 ml/min. The gradient started at 30% acetonitrile, and increased to 60% acetonitrile over 5 min. During further 1 min it was increased to 100% acetonitrile and maintained for 2 min before the gradient was returned to starting conditions in 1 min and kept there for 5 min. A 3 µl extract was injected for all samples. The mass spectrometer (MS) was operated in positive electrospray ionization mode and was automatically calibrated on the instrument ESI tuning mix (Agilent). The MS was used in selected ion monitoring (SIM) mode for the following $[M+H]^+$: FB_2 (m/z 706), FB_4 (m/z 690). The capillary voltage was held at 3000 V, the fragmentor voltage was at 70 V and the nebuliser pressure was at 2.5 bar. The drying gas flow was 12 l/min with a temperature of 350 °C. Selected extracts were verified by LC-DAD-HRMS (Frisvad et al., 2007).

2.6. Isolation and analysis of nucleic acids

The cultures used for the molecular studies were grown on malt peptone (MP) broth using 10% (v/v) of malt extract (Oxoid) and 0.1% (w/v) bacto peptone (Difco), 2 ml of medium in 15 ml tubes. The cultures were incubated at 25 °C for 7 days. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnology, Madison, Wisconsin) according to the instructions of the manufacturer. Amplifications of the partial calmodulin gene were set up as described previously (Hong et al., 2006). All the sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in double-distilled water and analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems) using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands, and the sequences were aligned with the MT Navigator software (Applied Biosystems).

3. Results and discussion

In total 47 black Aspergilli were isolated from the tea samples. The metabolite production of the 47 black Aspergilli was analyzed (Table 1). None of the 47 isolates was able to produce FB_2, FB_4 nor OTA. All of the 47 isolates produced asperazine and funalenone. In addition, production of pyranoningin A was observed in 38 of the isolates, naphto-γ-pyrones in 44 isolates and antafumicins in 12 isolates. According to the literature, *A. niger sensu stricto* is able to produce both funalenone, naphto-γ-pyrone and pyranoningin A, but lacks the ability to produce antafumicin and asperazine and this species was hereby excluded as being the species from the tea (Samson et al., 2007). Since *A. foeditus* has recently been synonymized with *A. niger* (Peterson 2008) only three members within section *Nigri* are able to produce asperazine. These are *Aspergillus acidus* (=*A. foetidus* var. *acidus*; (Kozakiewicz 1989)), *A. tubingensis* and *A. vadensis* (Samson et al., 2007). The latter, *A. vadensis*, is not able to produce either antafumicin, funalenone or pyranoningin A (Samson et al., 2007). However, 12 of the isolates were able to produce antafumicins, which are only produced by *A. acidus* (Samson et al., 2007). The last 35 isolates were hereby provisionally identified as *A. acidus* or *A. tubingensis*. To further help the identification process we examined sequences of part of the calmodulin gene in 17 isolates, randomly chosen from these 35 isolates to prove their species assignment. The partial calmodulin gene sequences of all the examined isolates were identical to that of the type strain of *A. acidus*, isolate CBS 564.65^T (=ITEM 4507=ATCC 16874=IMI 104688; Genbank accession number AM419749). All 35 isolates were hereby identified as *A. acidus*.

Even though we have a limited amount of samples the results from this preliminary study point to the fact that *A. acidus* is the dominant fungus in Puerh tea and black tea, in contrast to previous studies which describe *A. niger* as the dominant fungus in Puerh tea fermentation and in black tea (Abe et al., 2008; Bouakline et al., 2000; Mo et al., 2005; Sano et al., 1986; Xu et al., 2005). In these studies a broader concept of *A. niger* was used, which would include what is circumscribed as *A. acidus* today. This leads us to conclude that the reported black Aspergilli from Puerh tea and black tea probably should be re-identified as *A. acidus*. In our screening there was no indication of *A. niger sensu stricto* in any of the tea analyzed in this study. Since only *A. acidus* is found and this does not produce FB_2, FB_4 nor OTA, it is very unlikely that the production of these mycotoxins by *A. niger sensu stricto* can occur in tea. This correlates with the observation that among the fumonisins, only FB_1 from *Fusarium* spp. has been isolated from tea, and *A. niger* is not able to produce FB_1 (Frisvad et al., 2007; Martins et al., 2001).

To support this preliminary study, further investigation of more samples, as well as additional samples from different regions and stages in the tea processing should be performed. However, it is predicted that no or a very low amount of *A. niger* will be found in Puerh tea or other black teas, making any presence of FB_2, FB_4 and OTA from *A. niger* in this commodity less likely.

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References

Abarca, M.L., Bragulat, M.R., Castella, G., Cabañes, F.J., 1994. Ochratoxin A production by strains of *Aspergillus niger* var *niger*. Applied and Environmental Microbiology 60, 2650–2652.
Abe, M., Takaoka, N., Idemoto, Y., Takagi, C., Imai, T., Nakasaki, K., 2008. Characteristic fungi observed in the fermentation process for Puer tea. International Journal of Food Microbiology 124, 199–203.
Baker, S.E., 2006. *Aspergillus niger* genomics: past, present and into the future. Medical Mycology 44, S17–S21.
Bouakline, A., Lacroix, C., Roux, N., Gangneux, J.P., Derouin, F., 2000. Fungal contamination of food in hematology units. Journal of Clinical Microbiology 38, 4272–4273.
Elshafie, A.E., Al Lawatia, T., Al Bahry, S., 1999. Fungi associated with black tea and tea quality in the sultanate of Oman. Mycopathologia 145, 89–93.
Frisvad, J.C., Samson, R.A., 2004. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium* - A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Studies in Mycology 1–173.
Frisvad, J.C., Smedsgaard, J., Samson, R.A., Larsen, T.O., Thrane, U., 2007. Fumonisin B2 production by *Aspergillus niger*. Journal of Agricultural and Food Chemistry 55, 9727–9732.
Hocking, A.D., Pitt, J.I., 1980. Dichloran-Glycerol Medium for Enumeration of Xerophilic Fungi from Low-Moisture Foods. Applied and Environmental Microbiology 39, 488–492.
Hong, S.B., Cho, H.S., Shin, H.D., Frisvad, J.C., Samson, R.A., 2006. Novel *Neosartorya* species isolated from soil in Korea. International Journal of Systematic and Evolutionary Microbiology 56, 477–486.
Kozakiewicz, Z., 1989. *Aspergillus* species on stored products. Mycological Papers 161, 1–188.
Martins, M.L., Martins, H.M., Bernardo, F., 2001. Fumonisins B1 and B2 in black tea and medicinal plants. Journal of Food Protection 64, 1268–1270.
Mo, H.Z., Xu, X.Q., Yan, M.C., Zhu, Y., 2005. Microbiological analysis and antibacterial effects of the indigenous fermented Puer tea. Agro Food Industry Hi-Tech 16, 16–18.
Noonim, P., Mahakarnchanakul, W., Nielsen, K.F., Frisvad, J.C., Samson, R.A., 2009. Fumonisin B2 production by *Aspergillus niger* from Thai coffee beans. Food Additives and Contaminants 26, 94–100.
Pel, H.J., de Winde, J.H., Archer, D.B., Dyer, P.S., Hofmann, G., Schaap, P.J., Turner, G., de Vries, R.P., Albang, R., Albermann, K., Andersen, M.R., Bendtsen, J.D., Benen, J.A.E., van den Berg, M., Breestraat, S., Caddick, M.X., Contreras, R., Cornell, M., Coutinho, P.M., Danchin, E.G.J., Debets, A.J.M., Dekker, P., van Dijck, P.W.M., van Dijk, A., Dijkhuizen, L., Driessen, A.J.M., d'Enfert, C., Geysens, S., Goosen, C., Groot, G.S.P., de Groot, P.W.J., Guillemette, T., Henrissat, B., Herweijer, M., van den Hombergh, J.P.T.W., van den Hondel, C.A.M.J., van der Heijden, R. T.J.M., van der Kaaij, R.M., Klis, F.M., Kools, H.J., Kubicek, C.P., van Kuyk, P.A., Lauber, J., Lu, X., van der Maarel, M.J.E.C., Meulenberg, R., Menke, H., Mortimer, M.A., Nielsen, J., Oliver, S.G., Olsthoorn, M., Pal, K., van Peij, N.N.M.E., Ram, A.F.J., Rinas, U., Roubos, J.A., Sagt, C.M.J., Schmoll, M., Sun, J.B., Ussery, D., Varga, J., Vervecken, W., de Vondervoort, P.J.J.V., Wedler, H., Wosten, H.A.B., Zeng, A.P., van Ooyen, A.J.J., Visser, J., Stam, H., 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nature Biotechnology 25, 221–231.
Peterson, S.W., 2008. Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. Mycologia 100, 205–226.

Pitt, J.I., 1979. The Genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. London: Academic Press.

Samson, R.A., Houbraken, J.A.M.P., Kuijpers, A.F.A., Frank, J.M., Frisvad, J.C., 2004. New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*. Studies in Mycology 50, 45–61.

Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C., Varga, J., 2007. Diagnostic tools to identify black Aspergilli. Studies in Mycology 59, 129–145.

Sano, M., Takenaka, Y., Kojima, R., Saito, S., Tomita, I., Katou, M., Shibuya, S., 1986. Effects of Pu-Erh tea on lipid-metabolism in rats. Chemical & Pharmaceutical Bulletin 34, 221–228.

Xu, X., Yan, M., Zhu, Y., 2005. Influence of fungal fermentation on the development of volatile compounds in the Puer tea manufacturing process. Engineering in Life Sciences 5, 382–386.

Paper 6

”Studies on fumonisins in peanuts and peanut butter”

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Paper to be submitted to Journal of Agricultural and Food Chemistry

Studies on fumonisins in peanuts and peanut butter

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Abstract

Fumonisins are important carcinogenic mycotoxins, mainly associated to maize and other crops infected with *Fusarium* species. Recently *Aspergillus niger* contamination hase been shown to be the source of fumonisin contamination in grapes, raisins and wine, and we speculate if peanut derived products were also contaminated by fumonisins as *A. niger* is a very frequent contaminant of peanuts. Here we investigate the production of fumonisins on both peanuts artificially contaminated by *A. niger*, as well as retail peanuts and peanut butter. Fumonisins were quantified by isotope dilution LC-MS/MS after an-ion exchange purification. Fumonisins were detected in all artificially infected peanuts at levels up to 10 mg/kg whereas no fumonisins were detected in 15 peanut and 9 peanut butter retail products (limit of detection 0.004 mg/kg). We speculate that the absence of fumonisins in the retail products is a results of the severe damage induced to the nuts by *A. niger* which facilitates easily separation of infected and non-infected nuts.

Introduction

Mycotoxins produced by some fungal species, especially *Aspergillus*, *Penicillium* and *Fusarium* [1] are naturally occurring in many food and feed types. The most toxic and commonly occurring ones (aflatoxins, ochratoxin A, fumonisins, trichothecenes, and zearalenone) are subsequently regulated and routinely monitored.

The fumonisins (**Figure 1**), are mainly associated with *Fusarium* infected maize but however newer discoveries have shown that also *Aspergillus niger* and three *Tolypocladium* species produce these compounds [2-5]. While *Fusarium* species have a wide fumonisin profile showing FB_1-FB_5 as well as the A-, C- and P- groups, *A. niger* is only known to produce FB_2, FB_4 and FB_6 and *Tolypocladium* only FB_2 and FB_4 [2,3,5,6].

Since fumonisins are regularly found in toxic levels in especially maize products they are regulated in to levels of 0.2-4 mg/kg in US and EEC depending on the maize type and intended use [7,8].

A. niger is among the commonest fungi isolated from nuts, especially peanuts, and has also been reported in pecans, pistachios, hazelnuts, walnuts, coconut copra, cereals and oilseeds [9-14].

Infection of peanuts by *Aspergillus*, especially members of section *Flavi* and *Nigri* occurs under both pre-harvest and post-harvest conditions [15,16]. The main problem organisms concerning peanuts and mycotoxins are *A. flavus* and the related *A. parasiticus*, due to their production of the highly carcinogenic aflatoxins and the fact that they do not visually damage the nuts but only give some discoloration that can be further enhance after roasting [17]. Although, *A. niger* is also frequently isolated from peanuts it has until recently not been considered a safety problem as very few strains produce ochratoxin A. But with the discovery that75-80% of all strains, independent of isolation commodity, can produce fumonisins [2,3,18-21] peanuts is a subsequently potential source of fumonisin exposure.

45 In a project screening novel fumonisin contaminated food products we decided to: i) investigate if
46 *A. niger* can produce fumonisins while growing on peanuts and ii) develop a LC-MS/MS method for
47 quantification fumonisins in peanuts and peanut butter.
48
49 **Materials and Methods**
50 Unless otherwise stated, all solvents were high-performance liquid chromatography (HPLC)-grade
51 and other chemicals were analytical-grade from Sigma-Aldrich (St. Louis, MO). Water was purified
52 on a Milli-Q system (Millipore, Bedford, MA). The mixed certified standard of U-$^{13}C_{34}$-fumonisin B_2,
53 FB_1 and FB_2 were from Biopure (Tulln, Austria). Fumonisins FB_4 and FB_6 were available from
54 previous studies [4].
55
56 **Peanut samples**
57 Peanut samples (n=15) were purchased at local markets from three locations in Thailand (Surat
58 Thani, Chang Mai and Bangkok). Peanut butter samples were purchased from Danish
59 supermarkets and specialty shops. In total 9 brands were tested, they were produced in USA (n=2),
60 the Netherlands (origin unknown) (n=2), Argentina (n=2), Denmark (n=2) (origin: China) and
61 England (n=1) (origin: Indonesia).
62
63 **Inoculation of *A. niger* on peanuts**
64 Shelled peanuts were sterilized in 2% hypochlorite for 2 min, and washed twice in sterile water.
65 The peanuts were placed in a petri dish with water agar. A hole was punched in the shell with a
66 sterile needle (D=1-2 mm). Spore suspension (10 µL) of was placed upon the hole in the peanut
67 shell. The peanuts were then incubated at 25 ˚C for 7-14 days in darkness. Afterwards a single

peanut carefully removed from the shell and extracted. The following four fumonisin producing *A. niger* strains, were randomly selected from the IBT collection (Authors address): IBT 18741, 28753, 29019 and 29331. Unfortunately no *A. niger* strains originating from peanuts were available, we therefore randomly selected 4 strains from raisins.

Sample preparation

One shelled infected/control peanut (cut out of the shell) or 3 g retail peanuts were placed in a cryo tube with 10 steel balls and 3 ml methanol/water, and shaken for 5 minutes by a Mini Bead-beater (Biospec Products Inc, Bartlesville, OK). The peanut butter was considered homogeneous and 3 g peanut butter was extracted directly.

The 3 g retail peanuts (or single nuts) were extracted in total with 15 ml methanol/water (3:1 v/v) and 8 ml heptane, by thoroughly shaking for 15 minutes in a 50 ml Falcon tube on a shaking table at 200 RPM. Phases were separated by centrifugation at 9400 rpm for 5 minutes and the liquid middle phase (approx. 12 ml) from the resulting three-phase system was transferred to a new 50 ml falcon tube. The extract was further defatted by adding 8 ml heptane, vortexed and centrifuged at 9400 rpm for 5 minutes. The lower phase was transferred to a new falcon tube and excess heptane was removed after additional centrifugation. The pH was measured and adjusted to 6.3 ± 0.2 with formic acid or ammonium hydroxide. From each sample 9 ml was applied to a 200 mg/3 ml Strata SAX column (Phenomenex, Torrance, CA), which was previously conditioned with 1 ml water and 1 ml methanol. The cartridge was afterwards washed with 3 ml of methanol/water (3:1 v/v) and 2 ml methanol. Fumonisins were eluted with 2 ml methanol with 1 % formic acid and 20 ng U-$^{13}C_{34}$-fumonisin was added to each sample. Solvent was evaporated at 37 °C under a gentle

stream of nitrogen and redissolved in 2x100 µl acetonitrile/water (3:7 v/v) and used directly for analysis.

Validation

The fumonisin B_2 extraction were only validated on peanut butter. Validation was performed by spiking portions of 3 g peanut butter to the following concentrations of FB_2: 0 (blank), 0.004, 0.008, 0.016, 0.060, 0.25, 1, 4, 16 mg/kg. Three experiments of each validation were performed on 3 different days, with all levels in triplicates each day. To determinate the apparent recovery, the ratio of signal of FB_2 for spiked samples and standards was compared without U-$^{13}C_{34}$-FB_2 correction.

LC-MS/MS conditions

LC-MS/MS analysis was performed as previously described [20,22]. Briefly described the LC-MS/MS analysis was performed on a Quattro Ultima triple mass spectrometer (Micromass, Manchester, UK) using positive electrospray ionization. Separations was performed on a 50 × 2 mm i.d., 3 µm Gemini C6-phenyl column (Phenomenex, Torrance, CA) as described previously. One thing differentiated from the previously described method, an injection volume of 7 µL was used. Fumonisins B_2, B_4, B_1 and B_6 were monitored using two transitions each [20,22] while the internal standard, U-$^{13}C_{34}$-FB_2, was monitored by m/z 740 →358.

Results & Discussion

In order to get a robust defatting, several defatting agents were tested, e.g. ethyl acetate, heptane, pentane and toluene. Pentane evaporated too quickly, toluene gave unclear extraction

phase and ethyl acetate gave a lower recovery due to emulsion formation, therefore heptane was chosen. At first the defatting and extraction step was divided, but in order speed up the process, the extraction and defatting steps were combined in one step, with no significant drop in recovery. Two different extraction solvents were also tested, MeOH:H_2O (3:1 v/v) and ACN:H_2O (3:1 v/v). The 75% MeOH solution gave similar recovery as the 75% CAN, however ACN was deselected due to sedimentation (data not shown), and MeOH was chosen as the extraction solvent. Robustness testing of the fumonisin extraction at different pH values (5, 7, 9) gave the same recovery (data not shown).

Validation

The three calibration curves, one from each day, from spiked samples, had the following R^2 values: 0.9998, 0.9996, and 0.9999. The relative standard deviation (RSD) of the slope (peak area of quantifier/peak area-U-$^{13}C_{34}$ FB_2) from the 3 days varied within 4 and 17% (average 7%). Limit of quantification was 0.008 mg/kg (RSD: 16%) and limit of detection (s/n of 5:1) was 0.004 mg/kg. Absolute recovery of FB_2 was found to be 69 ± 14%.

Fungal growth on peanuts – Worst case scenarios

Since *A. niger* is a common contaminant of peanuts [11,13,16,23,24], we made worst case scenarios, to establish the potential fumonisin contamination. All four strains were able to produce fumonisins in peanuts (Table 1). Examples of *A. niger* growth on peanuts are given in Supporting Information 1.

Table 1: Production of Fumonisins by *Aspergillus niger* in Peanuts after 7-14 Days Growth at 25 °C

Incubation period	IBT 18741 (FB_2, mg/kg)		IBT 28753 (FB_2, mg/kg)		IBT 29019 (FB_2, mg/kg)		IBT 29331 (FB_2, mg/kg)	
	#1	#2	#1	#2	#1	#2	#1	#2
7 days	5.8	4.8	3.5	1.6	0.43	0.014	2.5	1.0
14 days	9.6	9.1	2.5	3.9	0.56	3.4	3.9	7.6

The mean production of fumonisin B_2 varied more than 40 fold from 0.22 mg/kg (IBT 29019) to 9.4 mg/kg (IBT 18741). Selected transitions from the LC-MS/MS analysis of infected and spiked peanuts are shown in the Supporting Information 2. The measured fumonisin concentrations (**Table 1**) are similar, compared to those detected in grapes and raisins (0.2-8 mg/kg) [19,20].

Peanut and peanut butter samples

Since *A. niger* produced fumonisins in high contaminations when growing on the peanuts we decided to screen peanut retail products. It was further decided to focus on peanut butter and peanuts purchased directly from local markets, as we assumed the quality would be lower than from the roasted and salted peanuts sold for snacks.

Analysis of 15 peanut samples (from local Thai markets) and 9 peanut butter brands (peanuts originating from 4 countries) all analyzed in triplicate (as well as a lot of samples during method development and validation) showed no indications of fumonisins (LOD: 0.004 mg/kg). Even though *A. niger* is commonly isolated from peanuts [9-14], and that when it is grown on the peanuts, fumonisins are produced in high quantities (up to 2000 higher than our detection limit), we suppose that the combination of: i) extensive damage to the nuts made by *A. niger* (in some cases they are heavily digested, see supporting information), and: ii) the high attention to reduce aflatoxin exposure [17] effectively removes *A. niger* infected peanuts and thus fumonisins. Altogether these results indicate that fumonisin exposure in peanuts is not health problem.

Supporting Information:

1: Example pictures of *A. niger* growth on peanuts.

2: LC-MS/MS chromatograms of spiked and non-spiked peanut samples.

Supporting information is available free of charge via the Internet at http://pubs.acs.org.

Acknowledgements

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Literature Cited

Reference List

1. **Sweeney, M. J.; Dobson, A. D. W. Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. *Int J Food Microbiol* 1998, *43* (3), 141-158.**

2. **Frisvad, J. C.; Smedsgaard, J.; Samson, R. A.; Larsen, T. O.; Thrane, U. Fumonisin B_2 production by *Aspergillus niger*. *J Agric Food Chem* 2007, *55* (23), 9727-9732.**

3. **Noonim, P.; Mahakarnchanakul, W.; Nielsen, K. F.; Frisvad, J. C.; Samson, R. A. Fumonisin B_2 production by *Aspergillus niger* from Thai coffee beans. *Food Addit Contam A* 2009, *26* (1), 94-100.**

4. **Månsson, M.; Klejnstrup, M. L.; Phipps, R. K.; Nielsen, K. F.; Frisvad, J. C.; Gotfredsen, C. H.; Larsen, T. O. Isolation and NMR characterization of fumonisin B_2 and a new fumonisin B_6 from *Aspergillus niger*. *J Agric Food Chem* 2010, *58*, 949-953.**

5. **Mogensen, J. M.; Moller, K. A.; von, F. P.; Labuda, R.; Varga, E.; Sulyok, M.; Kubatova, A.; Thrane, U.; Andersen, B.; Nielsen, K. F. Production of fumonisins B_2 and B_4 in *Tolypocladium* species. *J Ind. Microbiol Biotechnol.* 2011, *38*, 1329-1335.**

6. **Rheeder, J. P.; Marasas, W. F. O.; Vismer, H. F. Production of fumonisin analogs by *Fusarium* species. *Appl Environ Microbiol* 2002, *68* (5), 2101-2105.**

7. **U.S.Food and Drug Administration Guidance for Industry: Fumonisin levels in human foods and animal feeds. *United States Food and Drug Administration, Washington DC* 2001.**

8. **EEC. Setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. *Comm. Eur. Communities* 2007, *1126* (L255), 14-16.**

9. **Mphande, F. A.; Siame, B. A.; Taylor, J. E. Fungi, aflatoxins, and cyclopiazonic acid associated with peanut retailing in Botswana. *J Food Prot* 2004, *67* (1), 96-102.**

10. **Pitt, J. I.; Hocking, A. D. *Fungi and food spoilage;* 2nd ed.; Blackie Academic and Professional: London, U.K., 1997.**

11. **Youssef, M.; El-Maghraby, O.; Ibrahim, Y. Mycobiota and mycotoxins of egyptian peanut (*Arachis hypogeae* L.) seeds. *Int J Bot* 2008, *4* (4), ISSN.**

12. **Jimenez, M.; Mateo, R.; Querol, A.; Huerta, T.; Hernandez, E. Mycotoxins and mycotoxigenic molds in nuts and sunflower seeds for human consumption. *Mycopathologia* 1991, *115* (2), 121-127.**

13. **Ismail, M. A. Deterioration and spoilage of peanuts and desiccated coconuts from two sub-Saharan tropical East African countries due to the associated mycobiota and their degradative enzymes. *Mycopathologia* 2001, *150* (2), 67-84.**

14. **Pitt, J. I.; Hocking, A. D.; Bhudhasamai, K.; Miscamble, B. F.; Wheeler, K. A.; Tanboonek, P. The normal mycoflora of commodities from Thailand . 1. Nuts and oilseeds. *Int J Food Microbiol* 1993, *20* (4), 211-226.**

15. Klich, M. A. Aspergillus flavus: the major producer of aflatoxin. *Mol Plant Pathol* 2007, *8* (6), 713-722.

16. Magnoli, C.; Astoreca, A.; Ponsone, L.; Fernandez-Juri, M. G.; Chiacchiera, S.; Dalcero, A. Ochratoxin A and the occurrence of ochratoxin A-producing black aspergilli in stored peanut seeds from Cordoba, Argentina. *J Sci Food Agric* 2006, *86* (14), 2369-2373.

17. Pitt, J., I; Hocking, A. D. Mycotoxins in Australia: biocontrol of aflatoxin in peanuts. *Mycopathologia* 2006, *162* (3), 233-243.

18. Knudsen, P. B.; Mogensen, J. M.; Larsen, T. O.; Nielsen, K. F. Occurrence of fumonisins B_2 and B_4 in retail raisins. *J Agric. Food Chem.* 2010, *59*, 772-776.

19. Logrieco, A.; Ferracane, R.; Haidukowsky, M.; Cozzi, G.; Visconti, A.; Ritieni, A. Fumonisin B_2 production by *Aspergillus niger* from grapes and natural occurrence in must. *Food Addit Contam A* 2009, *26* (11), 1495-1500.

20. Mogensen, J. M.; Frisvad, J. C.; Thrane, U.; Nielsen, K. F. Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. *J Agric Food Chem* 2010, *58*, 954-958.

21. Pel, H. J.; de Winde, J. H.; Archer, D. B.; Dyer, P. S.; Hofmann, G.; Schaap, P. J.; Turner, G.; de Vries, R. P.; Albang, R.; Albermann, K.; Andersen, M. R.; Bendtsen, J. D.; Benen, J. A. E.; van den Berg, M.; Breestraat, S.; Caddick, M. X.; Contreras, R.; Cornell, M.; Coutinho, P. M.; Danchin, E. G. J.; Debets, A. J. M.; Dekker, P.; van Dijck, P. W. M.; van Dijk, A.; Dijkhuizen, L.; Driessen, A. J. M.; d'Enfert, C.; Geysens, S.; Goosen, C.; Groot, G. S. P.; de Groot, P. W. J.; Guillemette, T.; Henrissat, B.; Herweijer, M.; van den Hombergh, J. P. T. W.; van den Hondel, C. A. M. J.; van der Heijden, R. T. J. M.; van der Kaaij, R. M.; Klis, F. M.; Kools, H. J.; Kubicek, C. P.; van Kuyk, P. A.; Lauber, J.; Lu, X.; van der Maarel, M. J. E. C.; Meulenberg, R.; Menke, H.; Mortimer, M. A.; Nielsen, J.; Oliver, S. G.; Olsthoorn, M.; Pal, K.; van Peij, N. N. M. E.; Ram, A. F. J.; Rinas, U.; Roubos, J. A.; Sagt, C. M. J.; Schmoll, M.; Sun, J. B.; Ussery, D.; Varga, J.; Vervecken, W.; de Vondervoort, P. J. J. V.; Wedler, H.; Wosten, H. A. B.; Zeng, A. P.; van Ooyen, A. J. J.; Visser, J.; Stam, H. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat Biotechnol* 2007, *25* (2), 221-231.

22. Mogensen, J. M.; Larsen, T. O.; Nielsen, K. F. Widespread occurence of the mycotoxin fumonisin B_2 in wine. *J Agric Food Chem* 2010, *58*, 4853-4857.

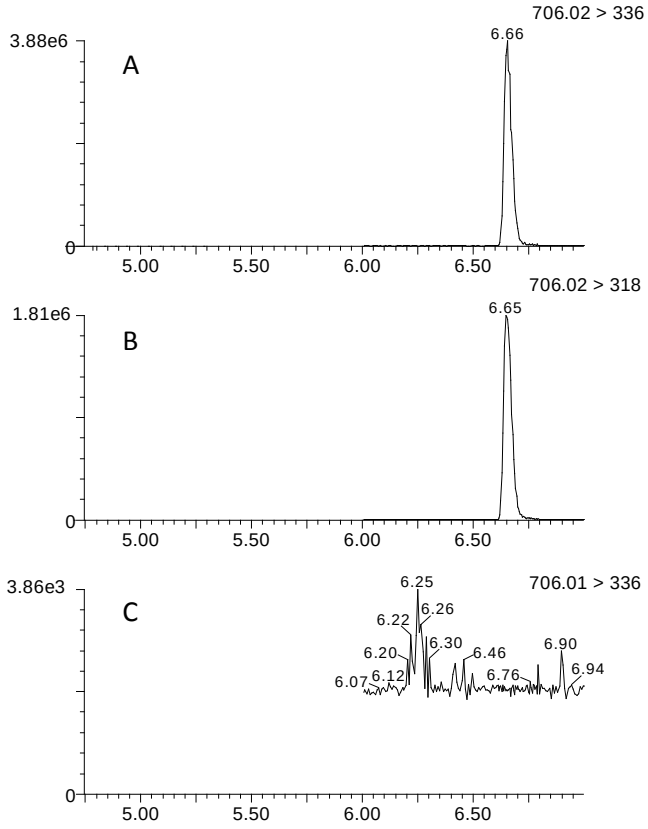
23. Horn, B. W. Relationship between soil densities of *Aspergillus* species and colonization of wounded peanut seeds. *Can J Microbiol* 2006, *52* (10), 951-960.

24. Magnoli, C.; Astoreca, A.; Ponsone, M. L.; Fernandez-Juri, M. G.; Barberis, C.; Dalcero, A. M. Ochratoxin A and *Aspergillus* section *Nigri* in peanut seeds at different months of storage in Cordoba, Argentina. *Int J Food Microbiol* 2007, *119* (3), 213-218.

Supporting information 1: Examples of *A. niger* growth on peanuts.

Supporting information 2:

MS/MS chromatograms of spiked and blank peanut samples. A: Qualifier of a spiked peanut sample (4 mg/kg). B: Quantifier of a spiked peanut sample (4 mg/kg). C: Qualifier of a peanut sample.



Paper 7

”Single kernel analysis of fumonisins and other fungal metabolites in maize from South African subsistence farmers”

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Single-kernel analysis of fumonisins and other fungal metabolites in maize from South African subsistence farmers

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Fumonisins are important *Fusarium* mycotoxins mainly found in maize and derived products. This study analysed maize from five subsistence farmers in the former Transkei region of South Africa. Farmers had sorted kernels into good and mouldy quality. A total of 400 kernels from 10 batches were analysed; of these 100 were visually characterised as uninfected and 300 as infected. Of the 400 kernels, 15% were contaminated with 1.84–1428 mg kg^{-1} fumonisins, and 4% ($n = 15$) had a fumonisin content above 100 mg kg^{-1}. None of the visually uninfected maize had detectable amounts of fumonisins. The total fumonisin concentration was 0.28–1.1 mg kg^{-1} for good-quality batches and 0.03–6.2 mg kg^{-1} for mouldy-quality batches. The high fumonisin content in the batches was apparently caused by a small number (4%) of highly contaminated kernels, and removal of these reduced the average fumonisin content by 71%. Of the 400 kernels, 80 were screened for 186 microbial metabolites by liquid chromatography-tandem mass spectrometry, detecting 17 other fungal metabolites, including fusaric acid, equisetin, fusaproliferin, beauvericin, cyclosporins, agroclavine, chanoclavine, rugulosin and emodin. Fusaric acid in samples without fumonisins indicated the possibility of using non-toxinogenic Fusaria as biocontrol agents to reduce fumonisin exposure, as done for *Aspergillus flavus*. This is the first report of mycotoxin profiling in single naturally infected maize kernels.

Keywords: cereals; fumonisins; LC/MS; mycology

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Introduction

Fumonisins are a group of naturally occurring, polyketide-derived mycotoxins produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum* (Rheeder et al. 2002). Recently, fumonisins B_2 and B_4 (FB_2, FB_4) were also detected in *Aspergillus niger* (Frisvad, Smedsgaard et al. 2007; Noonim et al. 2009) and *Tolypocladium* (Mogensen et al. 2011). Fumonisin production has also been detected in a single strain of *Alternaria arborescens* (as *Alternaria alternata* f. sp. *lycopersici*) (Chen et al. 1992) that has since lost this ability (Solfrizzo et al. 2005). Fumonisins are an important health risk because they are potentially carcinogenic and cause toxicoses in humans and animals (Sydenham et al. 1991). The effects of fumonisins include outbreaks of equine leukoencephalomalacia, and porcine pulmonary oedema and hydrothorax (Harrison et al. 1990; Thiel et al. 1991).

Fusarium species occur worldwide in maize, where they infect the cob during flowering. They can produce high amounts of fumonisins in tropical and subtropical regions (Shephard et al. 1996; Marasas 2001; Reddy et al. 2009) whereas in colder regions the fumonisin contamination is much lower (Logrieco et al. 2002; Miller 2008). In addition to maize-derived products, fumonisins have also been detected in rice (Yazar and Omurtag 2008), black tea leaves (Martins et al. 2001), asparagus (Logrieco et al. 1998) and pine nuts (Marin et al. 2007). Recently *Aspergillus niger* has been shown to be the source of fumonisins in wine and raisins (Knudsen et al. 2010; Logrieco et al. 2010; Mogensen, Larsen et al. 2010). Because of their ubiquitous natural occurrence in maize and maize-based foods (Shephard et al. 1996), fumonisins have attracted considerable global attention from food safety authorities. Consequently, international consultations and risk assessments have resulted in the United States Food and Drug Administration (USFDA) recommending a maximum level in maize for human consumption of 2–4 mg kg^{-1} total fumonisins depending on the maize product (USFDA 2001), and the European Union setting a regulatory limit of 0.2–2 mg kg^{-1} (European Union 2007).

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Maize is the staple cereal food grown and it is consumed by rural farming communities of Africa, especially in the Transkei region in South Africa (Shephard et al. 2002), where the effects of fumonisins were first discovered. The Transkei region has one of the highest incidences of oesophageal cancer in the world, which seems to be associated with fumonisin intake (Makaula et al. 1996; Shephard et al. 2002).

Analysis of single kernels has been performed previously with different methodologies, for instance: GC-MS for ergosterol in wheat kernels (Dong et al. 2006), ELISA for deoxynivalenol in wheat kernels (Sinha and Savard 1997), and DESI-MS for fumonisin in the surface of spiked maize kernels (Maragos and Busman 2010).

In this study we examined subsistence-grown maize from the former Transkei region of South Africa sorted into high- and low-quality cobs by the farmers themselves. We surveyed the fumonisin content of single maize kernels to establish the effects of manual visual sorting and determine the levels of fumonisin in the single kernel. Since numerous kernels appearing infected did not contain fumonisins, a further 20% of the analysed kernels were reanalysed for 186 microbial metabolites by a multi-toxin method to detect compounds from non-fumonisin producing Fusaria as well as post-harvest contaminants as Penicillia and Aspergilli.

Materials and methods

Unless otherwise stated, all solvents were high-performance liquid chromatography (HPLC) grade and other chemicals were analytical grade from Sigma-Aldrich (St. Louis, MO, USA). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Certified standard FB_1, FB_2 and FB_3 were from Biopure (Tulln, Austria). Standards of FB_4 and FB_6 were from previous studies (Månsson et al. 2010).

Maize samples

Ten batches of maize (approximately 500 g) were obtained from subsistence farmers in the Centane magisterial district of the former Transkei region, South Africa. Five batches had been classified by the farmers who grew them as high-quality cobs, good maize to be used for human consumption, and five as mouldy quality (mouldy) maize cobs. The mean percentage of infected/damaged kernels of each batch was determined by counting infected kernels in a random selection of 100.

Isolation and identification of mycoflora

Kernels were surface sterilised in 0.4% sodium hypochlorite for 2 min, then washed in sterile water. Six kernels were placed on each of the following media: dichloran 18% glycerol agar (DG18) (Hocking and Pitt 1980), Czapek iprodione dichloran agar (CZID) (Abildgren et al. 1987), and potato–carrot–manganese agar (PCMA) (Sørensen et al. 2009). In addition, six visibly infected or damaged kernels from each batch were placed on 2% water agar, resulting in an overall analysis of 24 kernels per batch and a total of 240 kernels. Plates were incubated for 7 days at 25°C in darkness. *Fusarium*, *Penicillium* and *Aspergillus* species were sub-cultured onto media appropriate for the genus and identified as previously described (Frisvad and Samson 2004; Samson et al. 2006, 2010; Frisvad, Larsen et al. 2007; Wulff et al. 2010), including secondary metabolite profiling and comparison to ex-type cultures (Nielsen and Smedsgaard 2003).

Extraction of kernels

For each maize batch, kernels were divided into groups of undamaged/uninfected and damaged/infected. Based on visual inspection of each batch, ten uninfected and 30 infected kernels were selected for chemical analysis, for a total of 400 kernels.

A single kernel was transferred into a 5-ml cryotube containing ten steel balls (diameter = 3 mm) and 1.5 ml distilled water added. The cryotubes were shaken for 5 min by a Mini Bead-beater (Biospec Products Inc., Bartlesville, OK, USA). Afterwards, 1.5 ml acetonitrile were added and the tubes were shaken on a shaking platform for 30 min. The mixture was centrifuged at 6000 g and 1.5 ml supernatant filtered through a 0.45-μm polytetrafluoroethylene filter and used directly for analysis.

LC-MS/MS fumonisin analysis

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed on a Quattro Ultima triple mass spectrometer (Micromass, Manchester, UK) with an electrospray ionisation (ESI) source as previously described (Mogensen, Frisvad et al. 2010). Separations were performed on a 50-mm Gemini C_6-phenyl column (Phenomenex, Torrance, CA, USA), using an acidic linear gradient from 20% acetonitrile to 55% in 6 min at a flow rate of 0.3 ml min^{-1}. Tandem MS was performed in ESI^+, with the MS in multiple reaction-monitoring (MRM) mode (Mogensen, Larsen et al. 2010; Mogensen, Frisvad et al. 2010). Transitions were: FB_2 and FB_3 m/z $706 \rightarrow 336$ and $706 \rightarrow 512$; FB_4 m/z $690 \rightarrow 320$ and $690 \rightarrow 514$; and FB_1 and FB_6 m/z $722 \rightarrow 334$ and

$722 \rightarrow 528$. A few extracts from kernels with the highest infection rate were additionally screened by LC-time-of-flight (TOF)-MS for other fumonisin analogues (Nielsen and Smedsgaard 2003).

Fumonisin extraction was validated by spiking single kernels of undamaged/uninfected maize. The kernels were split with a scalpel and spiked with fumonisin standards to final individual FB_1 and FB_2 concentrations of 6.7, 2.3, 0.75, 0.25 and $0.081\,mg\,kg^{-1}$. The kernels were air dried at room temperature for 1 h before extraction. Experiments were performed in triplicate on 3 different days. The apparent recovery was calculated from acetonitrile:water (1:1)-diluted samples with similar concentrations of fumonisins.

LC-MS/MS multi-mycotoxin screening

From the 400 extracts analysed for fumonisins, 80 were selected from the 10 batches to represent a combination of high, low and no amounts of fumonisins. The 80 samples (eight from visually non-infected kernels and 72 from visibly infected or damaged kernels) were analysed for 186 bacterial and fungal metabolites using a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray ESI source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany), as described by Vishwanath et al. (2009). Chromatographic separation was performed at 25°C on a 15 cm Gemini C_{18}-column. The chromatographic methods and chromatographic and MS parameters for the investigated analytes were as described by Vishwanath et al. (2009). ESI-MS/MS was performed in time-scheduled MRM mode in both positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte was set to its expected retention time ±30 s in positive mode and ±60 s in negative mode.

Results and discussion

Fungal infection rate of the maize batches

The good-quality batches all had a rate of infected/damaged kernels below 3% ($n = 100$), while the rate in mouldy batches was 6–19%, demonstrating a clear difference in maize quality (Table 1). The average mass of infected and uninfected kernels was 0.29 g and 0.37 g ($n = 14$ each) with a relative standard deviation (RSD) of 14% and 16%, respectively. The visibly infected or damaged kernels plated on water agar showed a 17–83% *Fusarium* infection rate (Table 1), with only fumonisin-producing Fusaria. Primarily only *F. subglutinans* and *F. verticillioides* were found, as previously found in South African maize (Rheeder et al. 1992; van der Westhuizen et al. 2011b). The less heat-tolerant *F. graminearum* was found only once among the randomly selected kernels. *Penicillium concavorugulosum* was found in four batches, *A. wentii* in three, *Eurotium* sp. in one, *P. aurantiogriseum* in two, *P. crustosum* in two, *P. pittii* in one and *P. brevicompactum* in one.

Validation of fumonisin extraction

The three calibration curves from different days had R^2-values of FB_1: 0.9932, 0.9986, 0.9974; and FB_2: 0.9974, 0.9978, 0.9984. The RSDs of the slopes were 7.8% (FB_1) and 16.1% (FB_2). The average RSD of the spiked sample ($n = 3$) for FB_1 was 12% (range = 7.5–19%) and for FB_2 17% (range = 3.4–27%). The limit of quantification (RSD < 20%) was $0.25\,mg\,kg^{-1}$ for FB_1 and $0.081\,mg\,kg^{-1}$ for FB_2. The limit of detection (LOD) was estimated to an s/n ratio of 5:1, giving an LOD_{FB1} of $0.16\,mg\,kg^{-1}$ and LOD_{FB2} of $0.057\,mg\,kg^{-1}$. The average apparent recovery for FB_1 was 121% with an average RSD of 25%, and for FB_2 was 115% with an average RSD of 20%.

Fumonisin content in single maize kernels

When single kernels were analysed for fumonisin, all ten batches contained positive kernels. Of the 400 tested kernels, 59 (15%) were positive for fumonisin. Of these, all were positive for FB_1, FB_2, FB_3 and FB_4, except two that did not contain FB_3 and FB_4. LC-TOF-MS screening of a few highly infected kernels also contained fumonisins A_1, A_2, C_1, C_3 and C_4, although in amounts much lower than FBs (data not shown).

None of the maize kernels that were visually characterised as uninfected contained detectable amounts of fumonisins, whereas 3–40% of the visually infected kernels were contaminated with fumonisins (Table 1). The total fumonisin concentration (FB_1, FB_2, FB_3 and FB_4) of single kernels in all the batches was $1.8–1428\,mg\,kg^{-1}$. Interestingly, the few low-quality kernels sorted from the good-quality cob batches generally had a higher fumonisin content compared with the poor-quality maize. However, kernels that were visually infected were more frequently found in the poor-quality cob batches.

Using the infection rate, average kernel weight, batch weight and average fumonisin concentration, the total fumonisin content in the batches was estimated. The estimated total fumonisin content of the good-quality batches was $0.28–1.1\,mg\,kg^{-1}$ (average = $0.71\,mg\,kg^{-1}$), while batches characterised as mouldy contained $0.03–6.2\,mg\,kg^{-1}$ fumonisins (average = $2.9\,mg\,kg^{-1}$). This showed a marked

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Table 1. Infection rate of batches and fumonisin (FB) content in uninfected and infected maize kernels ($n_{total} = 400$).

Batch	416	438	440	447	448	404	429	431	432	455
Quality[a]	Good	Good	Good	Good	Good	Mouldy	Mouldy	Mouldy	Mouldy	Mouldy
Infection rate (%)[b,c]	66	66	83	66	100	100	83	66	83	83
Dominating FB-producing fungi (%)[b,d]	*F. sub* (17)	*F. vert* (17), *F. sub* (17)	*F. vert* (17)	*F. vert* (50)	*F. sub* (33), *F. vert* (17)	*F. vert* (50), *F. sub* (33)	*F. vert* (17)	*F. vert* (17)	*F. sub* (33)	*F. vert* (17)
Other fungi[b,c,d]	*P. conca*, *A. wentii*, *P. auran*	*P. conca*, *P. auran*	*P. conca*	*A. wentii*		*Eurotium* sp.		*P. crust*, *A. wentii*	*P. pittii*, *P. brevi*	*P. conca*, *P. crust*
Visibly infected or damaged ($n = 100$) (%)	2	3	3	3	3	6	14	7	19	12
Uninfected kernels contaminated with FB ($n = 10$) (%)	0	0	0	0	0	0	0	0	0	0
Visually infected kernels contaminated with FB ($n = 30$) (%)	20	27	27	13	10	23	10	40	23	3
FB contents in infected kernels (minimum – maximum, $mg\,kg^{-1}$)	34.4–1428	4.0–90	1.9–715	1.8–432	5.8–929	2.1–257	39–736	3.2–968	4.6–713	8.4
Mean FB content in infected kernels ($mg\,kg^{-1}$)[e]	330	49	114	149	328	54.8	297	166	159	8.4
Median FB in infected kernels ($mg\,kg^{-1}$)	53	42	30	82	50	4.8	117	33	41	8.4
Total FB content ($mg\,kg^{-1}$)[f]	1.1	0.28	0.79	0.52	0.85	0.67	3.7	4.1	6.2	0.03

Notes: Fumonisin content is the total FB_1, FB_2, FB_3 and FB_4 content.
F. vert = *F. verticillioides*; *F. sub* = *F. subglutinans*; *P. auran* = *P. aurantiogriseum*; *P. brevi* = *P. brevicompactum*; *P. conca* = *P. concavurugulosum*; *P. crust* = *P. crustosum*.
[a]As sorted by the five subsistence farmers.
[b]Surface sterilisation in hypochlorite prior to plating for a total of 180 randomly selected kernels (18 per batch with six on DG18, six on CZID and six on PCMA).
[c]Plating of 60 visibly infected or damaged kernels on water agar per batch.
[d]Not including sterile mycelia and species found only once among the 240 kernels.
[e]LC-MS/MS on Ultima QQQ. LOD_{FB1} of $0.16\,mg\,kg^{-1}$ and LOD_{FB2} of $0.057\,mg\,kg^{-1}$.
[f]Total FB content was calculated from the infection rate, mean kernel weight, batch weight and mean fumonisin concentration.

difference in the fumonisin content of the high- and low-quality cob batches.

Of the ten batches, two exceeded the maximum level of $4\,mg\,kg^{-1}$ recommended by the USFDA in maize for human consumption (USFDA 2001), and four exceeded the European Union regulatory limit of $1\,mg\,kg^{-1}$ (European Union 2007). The values for fumonisin were similar to previous findings in South African subsistence maize (van der Westhuizen et al. 2008, 2010a). In a previous study van der Westhuizen et al. (2008) found levels of fumonisins in the mouldy maize (as classified on cobs by the farmers) that were higher (mean levels in different areas over two seasons $4.85–12.9\,mg\,kg^{-1}$) than those found in this study.

Multi-mycotoxin screening of selected kernels

In the previous analysis, the kernels were only tested for fumonisin, however since only 15% of the kernels were contaminated with fumonisin, we performed a multi-mycotoxin screening for identification of other toxins from non-fumonisin producing fungi. As well as to investigate the presence of compounds produced by post-harvest fungi, e.g. *P. concavorugulosum* and *A. wentii*.

Eighty kernels were selected and analysed by the multi-detection method for 186 bacterial and fungal metabolites, as described by Vishwanath et al. (2009). The results are shown in Table 2.

Fumonisins were detected in 50 of the 80 samples (Table 2), and only in four of the samples were no microbial metabolites detected. Other frequently detected metabolites were fusaric acid ($n=53$), emodin ($n=27$), chanoclavine ($n=25$), equisetin ($n=14$) and fusaproliferin ($n=12$). Metabolites found in a low number of samples were: agroclavine ($n=3$), altertoxin-I ($n=3$), beauvericin ($n=7$), moniliformin ($n=4$), nivalenol ($n=4$), rugulosin ($n=4$), and tentoxin ($n=3$). Other metabolites found in single cases were: alternariol, calphostin C, and cyclosporins A, C and H. To our knowledge, this is the first report of the fungal metabolites agroclavine, calphostin C, chanoclavine, cyclosporins, equisetin, rugulosin and tentoxin, as well as the bacterial metabolites nonactin, monactin and valinomycin in maize.

Table 3 shows a summary of Table 2 with the suspected producers of the metabolites as supported by mycological examination as well as if the compounds are produced pre- or post-harvest. Of the detected compounds and mycotoxins, many are presumably produced pre-harvest based on the natural field growth patterns of *Alternaria* and *Fusarium*. However, a lower number of toxins, generally considered to be produced post-harvest, were also detected, mostly those originating from *Penicillium*, *Aspergillus* and *Eurotium*. The finding of pre-harvest mycotoxins occurring from several Fusaria in the same maize kernels points toward the co-existence of the producers rather than species succession which could explain the remaining compounds originating from several species and genera.

Beauvericin, fusaproliferin and moniliformin (Table 1) are metabolites of *F. subglutinans*. Kernels with fusaproliferin and/or moniliformin often showed lower amounts of fumonisins, which could indicate the presence of *F. subglutinans*, which is known to be a poor fumonisin producer (Sala et al. 1994). Furthermore, samples containing fumonisin and fusaric acid had an average fusaric acid content of $37\,mg\,kg^{-1}$, while visually infected samples without fumonisin had a significantly higher average content of $112\,mg\,kg^{-1}$. Fusaric acid is a pre-harvest compound, a plant growth inhibitor and is suggested to be antifungal and antinematodic (Bacon et al. 1996; Diniz and Oliveira 2009). Its presence in samples without fumonisins strongly indicates that non-toxinogenic Fusaria were present in the maize, and supports the possibility of using these fungi as biocontrol agents against fumonisin-producing species, as is the case for *A. flavus* (Antilla and Cotty 2002; Cleveland et al. 2003; Abbas et al. 2006).

Equisetin is known to be produced by *F. equiseti*, which also produces nivalenol; however, this species was not detected. Nivalenol could also originate from *F. graminearum*, but this species was found only on a single kernel. However, the mycological screening was conducted on a limited number of kernels, so detection and isolation of a few *F. equiseti* or *F. graminearum* in samples dominated by *F. verticillioides* and *F. subglutinans* would have been difficult. In addition, different kernels were used for the mycological examination and for chemical analysis.

The cyclosporins could have originated from either *F. solani* or *Tolypocladium niveum*, both previously described as producers of cyclosporins (Dreyfuss et al. 1976; Isaac et al. 1990; Proksa et al. 1991; Sugiura et al. 1999).

Several *Alternaria* species can produce alternariol, tentoxin and altertoxin I, and the finding of these compounds was expected since *Alternaria* spp. grow superficially on many grain types (Andersen et al. 2002). Agroclavine, chanoclavine and rugulosin indicate that *P. concavorugulosum* (a known producer of these compounds; Frisvad 1989) was present in batches 429, 432, 438 and 440, which was validated by the mycological findings of this species these batches. Emodin is a common metabolite of many *Eurotium* species and *A. wentii* (Samson et al. 2010), which are common superficial contaminants on dried grain and were found on some kernels from two good and two mouldy batches. The presence of emodin and chanoclavine in the visually uninfected kernels was not surprising, as growth is superficial and thus easily

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Table 2. Fungal and bacterial metabolites in single maize kernels by multi-detection MS/MS analysis.

Batch	Maize quality[a]	Sample number	FB_1 ($mg\,kg^{-1}$)	FB_2 ($mg\,kg^{-1}$)	FB_3 ($mg\,kg^{-1}$)	FB_4 ($mg\,kg^{-1}$)	Fusaric acid ($mg\,kg^{-1}$)	Equisetin ($\mu g\,kg^{-1}$)	Chanoclavine ($\mu g\,kg^{-1}$)	Emodin ($\mu g\,kg^{-1}$)	Other compounds
416	Good	2486	3.03	0.53	0.38	0.17	1.9	–	–	–	
		2489	–	–	–	–	–	–	4.6	–	
		2491	94	39	8.5	8.8	8.1	68	–	–	
		2502	1910	866	167	127	7.3	55	–	–	Beauvericin, nivalenol
		2506	747	246	71	55	17	35	0.78	–	Beauvericin, nivalenol
438	Good	2527	9.3	2.9	1.1	0.57	17	–	0.53	–	
		2529	46	43	1.3	4.4	16	–	83	6.1	Agroclavine, rugulosin
		2531	21	8	2.9	2	12	–	–	9.2	Fusaproliferin, alternariol
		2533	–	0.14	–	–	46	–	–	18	Fusaproliferin
		2535	–	–	–	–	212	14	15	–	Fusaproliferin, monactin, nonactin, valinomycin
		2543	0.07	–	–	–	125	–	–	3.7	Altertoxin-I
		2545	–	–	–	–	–	7756	–	–	Fusaproliferin, beauvericin, nivalenol, tentoxin
		2546	149	26	6.8	4	26	–	0.52	–	Fusaproliferin
		2550	48	16	7.8	4.4	24	–	–	–	
440	Good	2558	–	–	–	–	0.2	–	–	7.3	
		2566	–	–	–	–	–	–	–	–	
		2567	0.18	0.062	–	–	–	218	1.3	–	
		2582	794	221	43	31	31	–	5.6	6.8	Rugulosin
		2584	4.7	1.2	0.61	0.34	63	–	6	–	
		2586	–	–	–	–	5.1	–	0.82	–	
		2591	37	11	4.2	2.9	12	–	–	35	
		2592	34	13	2.9	2.2	2.8	–	–	–	
447	Good	2603	–	0.033	–	–	–	–	–	–	
		2619	2.6	0.76	0.59	0.43	24	–	–	–	
		2622	–	–	–	–	–	–	–	–	
		2626	–	–	–	–	–	–	–	–	
		2629	730	203	47	26	1.7	–	–	46	Beauvericin
		2632	–	–	–	–	1.2	–	–	9.7	
		2633	–	–	–	–	–	–	–	–	
448	Good	2644	–	–	–	–	–	–	–	12	Beauvericin, tentoxin, altertoxin-I
		2647	5.6	0.26	0.6	–	4.7	1691	–	–	Moniliformin
		2649	1137	481	84	74	38	–	1	–	
		2652	0.58	0.08	–	–	–	–	–	–	Fusaproliferin, moniliformin
		2659	–	–	–	–	165	–	5.9	3.9	Fusaproliferin
		2665	–	–	–	–	73	12	–	–	
404	Mouldy	2681	–	–	–	–	–	1939	1.1	10	Cyclosporins A, C, H
		2687	206	72	13	11	25	–	3.8	–	
		2696	3	0.74	0.28	–	8.1	–	–	–	
		2699	1.8	0.57	0.2	0.0058	–	–	–	–	Fusaproliferin
		2704	–	–	–	–	40	–	–	–	

(*continued*)

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Table 2. Continued.

Batch	Maize quality[a]	Sample number	FB_1 (mg kg^{-1})	FB_2 (mg kg^{-1})	FB_3 (mg kg^{-1})	FB_4 (mg kg^{-1})	Fusaric acid (mg kg^{-1})	Equisetin (μg kg^{-1})	Chanoclavine (μg kg^{-1})	Emodin (μg kg^{-1})	Other compounds
		2706	–	0.038	–	–	–	–	–	–	
		2708	78	32	12	6.4	2.4	–	–	–	
429	Mouldy	2720	–	–	–	–	–	–	0.63	14	
		2727	–	–	–	–	1.0	–	–	36	
		2728	–	–	–	–	–	–	2.1	–	
		2730	886	385	20	20	4.4	–	–	–	
		2738	158	27	7.1	3.4	26	–	–	17	Rugulosin
		2741	–	–	–	–	–	–	–	16	
		2745	–	–	–	–	21	–	–	–	
431	Mouldy	2758	–	–	–	–	–	33	126	4.4	Agroclavine
		2766	1277	361	47	21	0.2	–	1.1	–	
		2767	2.4	0.64	0.25	0.0076	16	–	–	–	
		2768	8.5	1.9	0.61	0.39	0.4	–	0.59	–	
		2776	–	–	–	–	–	–	2.5	6.2	Fusaproliferin
		2781	–	–	–	–	23	24	1.5	–	
		2782	265	39	31	14	4.0	–	13	3.3	Fusaproliferin
		2784	932	254	47	34	55	–	–	–	Beauvericin, moniliformin
		2792	51	14	6.7	3.9	8.4	–	0.04	–	
432	Mouldy	2798	–	–	–	–	–	–	–	–	Nivalenol
		2807	0.43	0.13	–	–	0.8	–	106	75	Fusaproliferin, agroclavine, altertoxin-I, rugulosin
		2810	–	–	–	–	251	–	–	5.2	
		2815	–	0.041	–	–	–	–	–	–	
		2817	–	–	–	–	–	101	–	–	Tentoxin
		2820	30	22	4.6	9	23	–	–	–	
		2821	15	9	1.5	2.1	6.6	–	–	9.5	
		2822	–	–	–	–	–	–	–	14	Moniliformin
		2825	–	–	–	–	–	–	–	–	Calphostin C
		2826	739	306	83	92	13	24	–	–	
		2828	226	90	23	25	22	–	–	–	Beauvericin
		2829	3.5	2.1	0.31	0.32	17	–	–	–	
		2833	4	2.9	0.61	1.3	2.0	–	–	–	
455	Mouldy	2839	–	–	–	–	–	–	–	3.5	
		2847	0.47	0.35	0.14	0.18	0.6	–	–	8.9	
		2855	–	–	–	–	–	–	0.46	–	
		2856	1.1	0.16	–	–	49	–	–	–	
		2858	0.1	0.056	–	–	–	–	–	–	
		2860	5.9	1.2	0.74	0.35	29	–	–	–	
		2865	–	0.031	–	–	27	–	–	169	
		2868	–	0.036	–	–	–	709	–	58	Fusaproliferin
		2872	–	0.041	–	–	189	–	–	–	

Notes: '–' indicates that the levels were below the level of detection.
[a]As sorted by the five subsistence farmers.

Table 3. Distribution and likely producer of metabolites found in visually infected and uninfected kernels.

Mycotoxin	Percentage in uninfected kernels ($n=8$)	Percentage in infected kernels ($n=72$)	Likely producer	Supported by microbiology	Produced pre- or post-harvest
Alternariol	0	1	*Alternaria*	No	Pre[a]
Altertoxin-I	13	3	*Alternaria*	No	Pre[a]
Beauvericin	13	8	*F. subglutinans*	Yes	Pre
Calphostin C	0	1	*Cladosporium*	No	Pre[a]
Cyclosporin A, C and H	13	0	*F. solani* or *Tolypocladium*	No	Pre
Equisetin	25	17	*F. equiseti*	No	Pre
Fumonisin B_1	0	60	*F. verticillioides*, *F. subglutinans*	Yes	Pre
Fumonisin B_2	13	65	*F. verticillioides*, *F. subglutinans*	Yes	Pre
Fumonisin B_3	0	51	*F. verticillioides*, *F. subglutinans*	Yes	Pre
Fumonisin B_4	0	49	*F. verticillioides*, *F. subglutinans*	Yes	Pre
Fusaproliferin	0	17	*F. subglutinans*	Yes	Pre
Fusaric acid	13	64	*F. verticillioides*, *F. subglutinans* and other related	Yes	Pre
Moniliformin	0	6	*F. subglutinans*	Yes	Pre
Nivalenol	13	4	*F. equiseti*, *F. graminearum*	(Yes)[b]	Pre
Tentoxin	13	3	*Alternaria*	No	Pre[a]
Agroclavin	13	3	*P. concavurugulosum*	Yes	Post
Chanoclavine	38	31	*P. concavurugulosum*	Yes	Post
Emodin	75	29	*Eurotium*, *A. wentii*	Yes	Post
Rugulosin	0	6	*P. concavurugulosum*	Yes	Post

Notes: [a]As superficial growth usually close to harvest.
[b]*F. graminearum* was only found on a single kernel.

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abraded off the kernels so they appear uninfected. The bacterial metabolites monactin, nonactin and valinomycin were found only once, and in the same sample.

Even though aflatoxins, like fumonisins, are considered important mycotoxins in African maize, no aflatoxins were found in any of the analysed samples (Shephard 2008). These findings corresponded to the fungal isolation results, which found neither *A. flavus* nor *A. parasiticus* from any of the 240 kernels surveyed and also to a previous study conducted in the former Transkei region which also did not find any aflatoxin or *A. flavus* (van der Westhuizen et al. 2011a).

This is the first study to examine the mycotoxin profile of single maize kernels. The maize mycotoxin profiles were diverse, and the detection of metabolites from several fungi on single kernels supports the fact that several fungal species can co-occur on a single kernel. The results showed a low percentage of maize kernels containing fumonisins (15%), and an even lower percentage ($<4\%$) with a high concentration of fumonisins ($>100\,mg\,kg^{-1}$). The results also confirm that *Fusarium* mycotoxins are non-homogeneously distributed in maize (van der Westhuizen et al. 2011a). Although fumonisins appeared to be the cause of the most serious mycotoxin problem since they were the most widespread of the analysed mycotoxins, the fumonisin contamination in the maize samples was mainly caused by a low percentage of highly infected kernels. This supports the approach if a more thorough sorting of the subsistence grown maize is performed to decrease the fumonisin concentration. A theoretical calculation of the effect of removing the highly infected kernels (4%) showed that the average fumonisin concentration decreased by 71% (data not shown) after a simple sorting. This is a substantial effect gained using a very simple method. The strategy of sorting out visibly infected kernels has recently been successfully applied in an intervention study in the same rural Transkei area (van der Westhuizen et al. 2011b). A group of farmers were trained to remove infected kernels from their good-quality maize and achieved a mean fumonisin reduction of 84% by removing a mean of 3.9% by weight, which is very close to our estimated reduction (van der Westhuizen et al. 2010b, 2011a, b).

A valuable addition to these results could be to investigate the mycotoxin chemistry in single kernels, while simultaneously conducting an investigation of the mycology of the same kernel, for a better understanding of how species co-exist and how they are spread across the cob. This could also determine whether several fungal species infect the same kernel or if kernels are infected with only one species. Furthermore, data on single-kernel mycotoxin content may help in designing sampling plans for detecting mycotoxins in large lots. For example, Whitaker et al. (1972) suggested using a negative binominal

distribution and Vargas et al. (2005) showed that approximately six coffee beans per 10,000 beans are contaminated if a lot contains $5\,\mu g\,kg^{-1}$ of ochratoxin A. The results of our single-kernel mycotoxin analysis give an in-depth view of the extent of mycotoxin-producing fungi in South African Transkei subsistence maize, particularly fumonisins. These findings are valuable for designing sampling plans for large maize lots.

The results support the visual inspection method currently used by the maize-producing farmers and support additional sorting for even more effective removal of mycotoxin-contaminated kernels. Additional mycotoxin reduction might be achieved through biocontrol measures using a non-fumonisin-producing *Fusarium* strain. Finally, the single-kernel approach is promising for the detection of new fungal compounds in maize, as the concentrations of fungal compounds are much higher in the few infected kernels.

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References

Abbas HK, Zablotowicz RM, Bruns HA, Abel CA. 2006. Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillus flavus* isolates. Biocontrol Sci Techn. 16:437–449.

Abildgren MP, Lund F, Thrane U, Elmholt S. 1987. Czapek–Dox agar containing iprodione and dicloran as a selective medium for the isolation of *Fusarium* species. Lett Appl Microbiol. 5:83–86.

Andersen B, Krøger E, Roberts RG. 2002. Chemical and morphological segregation of *Alternaria arborescens, A. infectoria* and *A. tenuissima* species-groups. Mycol Res. 106:170–182.

Antilla L, Cotty PJ. 2002. The ARS-ACRPC partnership to control aflatoxin in Arizona: current status. Mycopathologia. 155:64.

Bacon CW, Porter JK, Norred WP, Leslie JF. 1996. Production of fusaric acid by *Fusarium* species. Appl Environ Microbiol. 62:4039–4043.

Chen JP, Mirocha CJ, Xie WP, Hogge L, Olson D. 1992. Production of the mycotoxin fumonisin B_1 by *Alternaria alternata* f. sp. *lycopersici*. Appl Environ Microbiol. 58:3928–3931.

Cleveland TE, Dowd PF, Desjardins AE, Bhatnagar D, Cotty PJ. 2003. United States Department of Agriculture – Agricultural Research Service research on pre-harvest prevention of mycotoxins and mycotoxigenic fungi in US crops. Pest Manag Sci. 59:629–642.

Diniz SPSS, Oliveira RC. 2009. Effects of fusaric acid on *Zea mays* L. seedlings. Phyton – Int J Exp Bot. 78:155–160.

Dong YH, Steffenson BJ, Mirocha CJ. 2006. Analysis of ergosterol in single kernel and ground grain by gas chromatography-mass spectrometry. J Agric Food Chem. 54:4121–4125.

Dreyfuss M, Harri E, Hofmann H, Kobel H, Pache W, Tscherter H. 1976. Cyclosporin-A and C new metabolites from *Trichoderma polysporum* (Link ex Pers.) Rifai. Eur J Appl Microbiol. 3:125–133.

European Union. 2007. Commission Regulation 2007/1126/EC of 28 September 2007 amending Regulation (EC) 1881/2006 setting maximum levels for certain contaminants in foodstuffs, regarding *Fusarium* toxins in maize and maize products. Off J Eur Comm. L 255/14–17.

Frisvad JC. 1989. The connection between the Penicillia and Aspergilli and mycotoxins with special emphasis on misidentified isolates. Arch Environ Con Tox. 18:452–467.

Frisvad JC, Larsen TO, de Vries R, Meijer M, Houbraken J, Cabanes FJ, Ehrlich K, Samson RA. 2007. Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins. Stud Mycol. 59:31–37.

Frisvad JC, Samson RA. 2004. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium* – a guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Stud Mycol. 49:1–173.

Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U. 2007. Fumonisin B_2 production by *Aspergillus niger*. J Agric Food Chem. 55:9727–9732.

Harrison LR, Colvin BM, Greene JT, Newman LE, Cole Jr JR. 1990. Pulmonary edema and hydrothorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium* moniliforme. J Vet Diagn Invest. 2:217–221.

Hocking AD, Pitt JI. 1980. Dichloran glycerol medium for enumeration of xerophilic fungi from low moisture foods. Appl Environ Microbiol. 39:488–492.

Isaac CE, Jones A, Pickard MA. 1990. Production of cyclosporins by *Tolypocladium niveum* strains. Antimicrob Agents Ch. 34:121–127.

Knudsen PB, Mogensen JM, Larsen TO, Nielsen KF. 2011. Occurrence of fumonisins B_2 and B_4 in retail raisins. J Agric Food Chem. 59:772–776.

Logrieco A, Doko B, Moretti A, Frisullo S, Visconti A. 1998. Occurrence of fumonisin B_1 and B_2 in *Fusarium proliferatum* infected asparagus plants. J Agric Food Chem. 46:5201–5204.

Logrieco A, Ferracane R, Visconti A, Ritieni A. 2010. Natural occurrence of fumonisin B_2 in red wine from Italy. Food Addit Contam A. 27:1136–1141.

Logrieco A, Mule G, Moretti A, Bottalico A. 2002. Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. Eur J Plant Pathol. 108:597–609.

Makaula AN, Marasas WF, Venter FS, Badenhorst CJ, Bradshaw D, Swanevelder S. 1996. Oesophageal and other cancer patterns in four selected districts of the Transkei, Southern Africa: 1985–1990. Afr J Health Sci. 3:11–15.

Månsson M, Klejnstrup ML, Phipps RK, Nielsen KF, Frisvad JC, Gotfredsen CH, Larsen TO. 2010. Isolation and NMR characterization of fumonisin B_2

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and a new fumonisin B_6 from *Aspergillus niger*. J Agric Food Chem. 58:949–953.

Maragos C, Busman M. 2010. Rapid and advanced tools for mycotoxin analysis: a review. Food Addit Contam A. 27:688–700.

Marasas WFO. 2001. Discovery and occurrence of the fumonisins: a historical perspective. Environ Health Perspect. 109:239–243.

Marin S, Ramos AJ, Vazquez C, Sanchis V. 2007. Contamination of pine nuts by fumonisin produced by strains of *Fusarium proliferatum* isolated from *Pinus pinea*. Lett Appl Microbiol. 44:68–72.

Martins ML, Martins HM, Bernardo F. 2001. Fumonisins B_1 and B_2 in black tea and medicinal plants. J Food Prot. 64:1268–1270.

Miller JD. 2008. Mycotoxins in small grains and maize: old problems, new challenges. Food Addit Contam A. 25:219–230.

Mogensen JM, Frisvad JC, Thrane U, Nielsen KF. 2010. Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. J Agric Food Chem. 58:954–958.

Mogensen JM, Larsen TO, Nielsen KF. 2010. Widespread occurrence of the mycotoxin fumonisin B_2 in wine. J Agric Food Chem. 58:4853–4857.

Mogensen JM, Møller KA, von Freiesleben P, Labuda R, Varga E, Sulyok M, Kubatova A, Thrane U, Andersen B, Nielsen KF. 2010. Production of fumonisins B_2 and B_4 in *Tolypocladium* species. J Ind Microbiol Biotechnol. 38:1329–1335.

Nielsen KF, Smedsgaard J. 2003. Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography-UV-mass spectrometry methodology. J Chromatogr A. 1002:111–136.

Noonim P, Mahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA. 2009. Fumonisin B_2 production by *Aspergillus niger* from Thai coffee beans. Food Addit Contam A. 26:94–100.

Proksa B, Uhrin D, Kovacik V, Voticky Z, Betina V. 1991. Identity of the antibiotic ramihyphin A and cyclosporine A. Folia Microbiol. 36:141–143.

Reddy KRN, Abbas HK, Abel CA, Shier WT, Oliveira CAF, Raghavender CR. 2009. Mycotoxin contamination of commercially important agricultural commodities. Toxin Rev. 28:154–168.

Rheeder JP, Marasas WFO, Thiel PG, Sydenham EW, Shephard GS, Vanschalkwyk DJ. 1992. *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. Phytopathology. 82:353–357.

Rheeder JP, Marasas WFO, Vismer HF. 2002. Production of fumonisin analogs by *Fusarium* species. Appl Environ Microbiol. 68:2101–2105.

Sala N, Sanchis V, Vilaro P, Viladrich R, Torres M, Vinas I, Canela R. 1994. Fumonisin producing capacity of *Fusarium* strains isolated from cereals in Spain. J Food Prot. 57:915–917.

Samson RA, Hong SB, Frisvad JC. 2006. Old and new concepts of species differentiation in *Aspergillus*. Med Mycol. 44:S133–S148.

Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B. 2010. Food and indoor fungi. Utrecht (the Netherlands): CBS-KNAW Fungal Biodiversity Centre.

Shephard GS. 2008. Risk assessment of aflatoxins in food in Africa. Food Addit Contam A. 25:1246–1256.

Shephard GS, Leggott NL, Stockenstrom S, Somdyala NIM, Marasas WFO. 2002. Preparation of South African maize porridge: effect on fumonisin mycotoxin levels. S Afr J Sci. 98:393–396.

Shephard GS, Thiel PG, Stockenstrom S, Sydenham EW. 1996. Worldwide survey of fumonisin contamination of corn and corn-based products. J AOAC Int. 79:671–687.

Sinha RC, Savard ME. 1997. Concentration of deoxynivalenol in single kernels and various tissues of wheat heads. Can J Plant Pathol. 19:8–12.

Solfrizzo M, De Girolamo A, Vitti C, Tylkowska K, Grabarkiewicz-Szczesna J, Szopinska D, Dorna H. 2005. Toxigenic profile of *Alternaria alternata* and *Alternaria radicina* occurring on umbelliferous plants. Food Addit Contam A. 22:302–308.

Sørensen JL, Mogensen JM, Thrane U, Andersen B. 2009. Potato carrot agar with manganese as an isolation medium for *Alternaria*, *Epicoccum* and *Phoma*. Int J Food Microbiol. 130:22–26.

Sugiura Y, Barr JR, Barr DB, Brock JW, Elie CM, Ueno Y, Patterson DG, Potter ME, Reiss E. 1999. Physiological characteristics and mycotoxins of human clinical isolates of *Fusarium* species. Mycol Res. 103:1462–1468.

Sydenham EW, Shephard GS, Thiel PG, Marasas WFO, Stockenstrom S. 1991. Fumonisin contamination of commercial corn-based human foodstuffs. J Agric Food Chem. 39:2014–2018.

Thiel PG, Shephard GS, Sydenham EW, Marasas WFO, Nelson PE, Wilson TM. 1991. Levels of fumonisin B_1 and fumonisin B_2 in feeds associated with confirmed cases of equine leukoencephalomalacia. J Agric Food Chem. 39:109–111.

United States Food and Drug Administration (USFDA). 2001. Guidance for industry: Fumonisin levels in human foods and animal feeds. Washington (DC): USFDA.

van der Westhuizen L, Shephard GS, Rheeder JP, Burger HM. 2010a. Individual fumonisin exposure and sphingoid base levels in rural populations consuming maize in South Africa. Food Chem Toxicol. 48:1698–1703.

van der Westhuizen L, Shepard GS, Rheeder JP, Burger HM, Gelderblom WCA, Wild CP, Gong YY. 2011a. Fumonisin B_1 as a urinary biomarker of exposure in a maize intervention study among South African subsistence farmers. Cancer Epidem Biomar. 20:483–489.

van der Westhuizen L, Shephard GS, Rheeder JP, Burger HM, Gelderblom WCA, Wild CP, Gong YY. 2011b. Optimising sorting and washing of home-grown maize to reduce fumonisin contamination under laboratory-controlled conditions. Food Contr. 22:396–400.

van der Westhuizen L, Shephard GS, Rheeder JP, Burger HM, Gelderblom WCA, Wild CP, Gong YY. 2010b. Simple intervention method to reduce fumonisin exposure in a subsistence maize-farming community in South Africa. Food Addit Contam A. 27:1582–1588.

van der Westhuizen L, Shephard GS, Rheeder JP, Somdyala NIM, Marasas WFO. 2008. Sphingoid base

levels in humans consuming fumonisin-contaminated maize in rural areas of the former Transkei, South Africa: a cross-sectional study. Food Addit Contam A. 25:1385–1391.

Vargas EA, Whitaker TB, dos Santos EA, Slate AB, Lima FB, Franca RCA. 2005. Testing green coffee for ochratoxin A, Part II: Observed distribution of ochratoxin A test results. J AOAC Int. 88:780–787.

Vishwanath V, Sulyok M, Labuda R, Bicker W, Krska R. 2009. Simultaneous determination of 186 fungal and bacterial metabolites in indoor matrices by liquid chromatography/tandem mass spectrometry. Anal Bioanal Chem. 395:1355–1372.

Whitaker TB, Wiser EH, Dickens JW, Monroe RJ. 1972. Comparison of observed distribution of aflatoxin in shelled peanuts to negative binomial distribution. J Am Oil Chem Soc. 49:590–593.

Wulff EG, Sørensen JL, Lübeck M, Nielsen KF, Thrane U, Torp J. 2010. *Fusarium* spp. associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity. Environ Microbiol. 12:649–657.

Yazar S, Omurtag GZ. 2008. Fumonisins, trichothecenes and zearalenone in cereals. Int J Mol Sci. 9:2062–2090.

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Paper 8

”Production of Fumonisins B_2 and B_4 in *Tolypocladium* species”

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ORIGINAL PAPER

Production of fumonisins B_2 and B_4 in *Tolypocladium* species

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Abstract *Tolypocladium inflatum* is known primarily for its production of the cyclosporines that are used as an immunosuppressive drug. However, we report here the production of the carcinogenic fumonisins B_2 and B_4 by this biotechnologically relevant fungal genus. These mycotoxins were detected in 11 strains tested from three species: *Tolypocladium inflatum*, *T. cylindrosporum*, and *T. geodes*. Production of fumonisins by *Fusarium* spp. and *Aspergillus niger* is highly medium- and temperature-dependent, so the effect of these parameters on fumonisin production by three *T. inflatum* strains was studied. Maximum production was achieved on media with high sugar content incubated at 25–30°C. Since these results demonstrate that fumonisin production could be widespread within the genus *Tolypocladium*, the potential contamination of commercial cyclosporine preparations with fumonisins needs to be investigated.

Keywords Fumonisin · Cyclosporin · Mycotoxin · *Tolypocladium inflatum* · *Elaphocordyceps subsessilis*

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Introduction

Species in the fungal genus *Tolypocladium* are found worldwide as soil-borne insect pathogens and saprotrophs [6, 35]. *Tolypocladium inflatum* W. Gams (syn. *T. niveum* [Rostr.] Bissett) is the best-known species, because it produces the cyclosporines that are used worldwide as an immunosuppressive drug in organ transplant recipients. In addition, the species produces antifungal and insecticidal efrapeptins [4, 16, 18]. *T. inflatum*, an anamorph of *Elaphocordyceps subsessilis* [Petch] G. H. Sung, J. M. Sung and Spatafora), is currently classified within *Ascomycota, Sordariomycetes, Hypocreales*, *Ophiocordycipitaceae* [41].

Fumonisins (Fig. 1) are regulated mycotoxins that cause human and animal toxicoses when consumed via contaminated maize-based food and feeds [46]. Fumonisin B_1 (FB_1) has furthermore been implicated as a risk factor for neural tube defects in embryos [21, 45]. Fumonisins were first discovered in *Fusarium verticillioides* (Sacc.) Nirenberg [5] and are a significant problem in maize-based products [14, 36, 39, 42]. Fumonisins are also occasionally found in rice [31], black tea leaves [23], asparagus [19], pine nuts [22], and wine [25]. The US Food and Drug Administration recommends that maize should not be used for human consumption at levels above 2–4 ppm total fumonisin [43] while the European Union (EU) has a regulatory limit of 0.2–2 ppm in maize products [10]. *Fusarium*

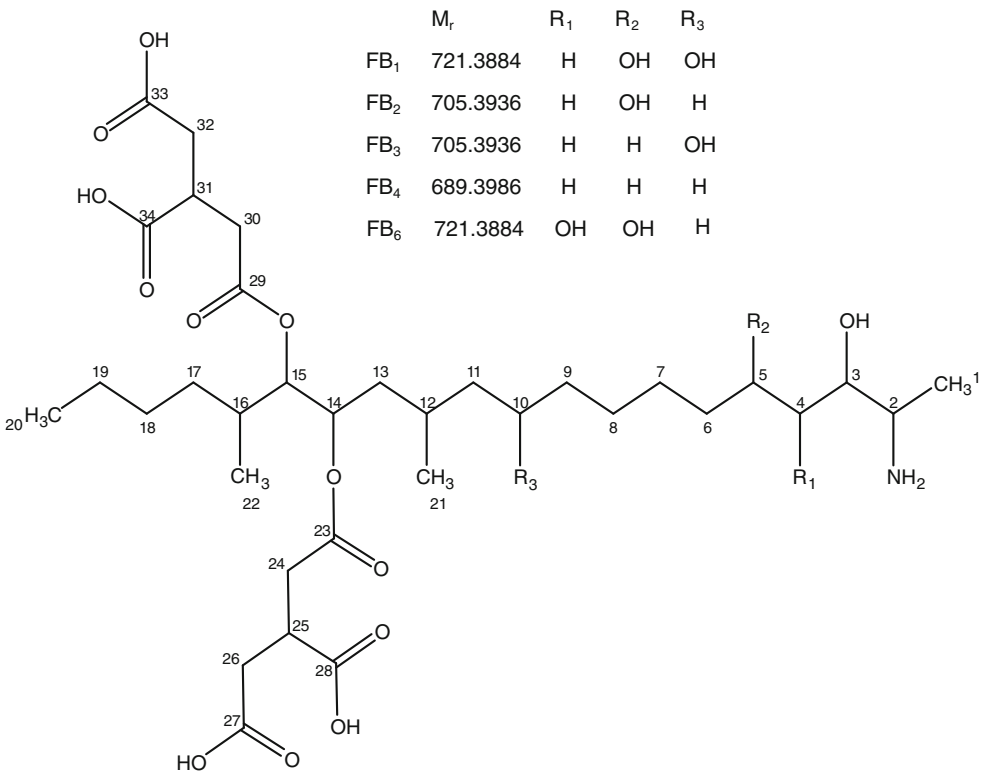

Fig. 1 Structure of fumonisins B_1, B_2, B_3, B_4 and B_6

is mainly the genus associated with fumonisin production, but a homolog to the *F. verticillioides* fumonisin gene cluster has been found in *Aspergillus niger* Tiegh. [1, 13, 20, 30, 32] and in a single strain of *Alternaria arborescens* E.G. Simmons (referred to as *Alternaria alternata* (Fr.) Kiessl. f. sp. *lycopersici* Grogan, Kimble & Misaghi in [47]). However, of the two only *A. niger* is able to produce fumonisins [13, 20, 30, 37].

During a routine screening of cyclosporine-producing fungi with the multi-detection method described by Vishwanath et al. [44], we were surprised to discover that a *Tolypocladium* strain was able to produce fumonisins. Since this has a potentially important impact on drug safety and genetic analysis, this study was initiated to survey the distribution of fumonisin production in *Tolypocladium* and to examine the influence of media and temperature on fumonisin production by species of this genus.

Materials and methods

Unless otherwise stated all solvents were HPLC grade, chemicals were analytical grade, and water was purified on a Milli-Q system (Millipore, Bedford, MA). Certified reference standards of fumonisin (B_1, B_2, and B_3) were from Romer Labs (Tulln, Austria), and FB_4 and FB_6 were purified from *A. niger* and validated by nuclear magnetic resonance (NMR) spectroscopy at the Technical University of Denmark [20].

Fungal strains, media, and incubation

Eleven *Tolypocladium* strains and one *Aspergillus niger* strain (IBT 28144 = CBS 101705, NRRL 567, ITEM 7097) were used in this study (see Table 1). To analyze the effect of media on the production of fumonisins ten media were used: Czapek yeast autolysate agar (CYA) [12], Czapek yeast autolysate agar + 5% NaCl (CYAS) [12], dichloran Rose bengal yeast extract sucrose agar (DRYES) [11], potato carrot agar (PCA) [37], malt extract agar (MEA according to Blakeslee) [34], oatmeal agar (OAT) [12], potato dextrose agar (PDA, Difco), V8-juice agar with antibiotics (V8) [37], yeast extract sucrose agar (YES) [12], and dichloran 18% glycerol agar (DG18) [15]. The composition of the media is given in Supplementary Table 1. Media were prepared in 9-cm Petri dishes, each with 17 mL medium, and strains were inoculated as three-point inoculations. Petri dishes were incubated in micro-perforated plastic bags at 25°C in the dark. All samples were analyzed in triplicate on three individual plates. Colony diameter measurements are given as averages of 9 colonies on 3 plates. To analyze the effect of temperature on the production of fumonisins in *T. inflatum*, three strains (IBT 41581, IBT 41582 and IBT 41583) were inoculated

Table 1 Production of fumonisins B_2 (FB_2) and B_4 (FB_4) by *Tolypocladium cylindrosporum*, *T. geodes*, and *T. inflatum*

Species	Strain number	FB_2	FB_4
T. cylindrosporum	CCF 1450	+	+
	CCF 2531	+	+
	CCF 3237	+	+
T. geodes	CCF 2548	+	−
	CCF 2579	+	+
	CCF 3299	+	−
T. inflatum	DSM 915	+	+
	DSM 63544	+	+
	IBT 41581	+	+
	IBT 41582	+	+
	IBT 41583	+	+

CCF Culture Collection of Fungi (Prague, Czech Republic); *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany); *IBT* Culture collection at Center for Microbial Biotechnology, DTU Systems Biology (Kgs. Lyngby, Denmark)

on YES and incubated at 15, 20, 25, 30, or 37°C in the dark for 7 days.

Metabolite extraction

Fumonisins were extracted by using the method previously described by Frisvad et al. [13]. Six plugs (6 mm diameter) were cut across the colony diameter through the center. If colony diameters were smaller than 20 mm, plugs were collected from two or three colonies from the plate. The six plugs were transferred to a 2-mL vial, 800 µL of methanol/water (3:1 v/v) was added, and vials were placed in an ultrasonication bath for 1 h. All extracts were filtered through a PTFE 0.45-µm syringe filter (National Scientific, Rockwood, Tennessee) into a new vial and used directly for analysis. Samples were made in triplicate from individual plates. This method was validated as in our previous reports with similar recovery of 75–85% [26, 28].

LC–MS/MS analysis of fumonisins

Fumonisins were analyzed by using liquid chromatography tandem mass spectrometry (LC–MS/MS) as previously described [24] with minor changes. The LC–MS/MS analyses were performed on a Quattro Ultima triple mass spectrometer (Micromass, Manchester, UK) with electrospray ionization (ESI) source, and separations performed on a 50 × 2 mm i.d., 3-µm Gemini C_6-phenyl column (Phenomenex, Torrance, California). A linear gradient was performed from 20% acetonitrile in water with 20 mM formic acid to 55% acetonitrile for 6 min at 0.3 mL/min, increasing to 100% acetonitrile in 30 s at 0.5 mL/min; this was maintained for 3.5 min before returning to the starting conditions in 6 min. Tandem MS was performed in ESI^+ with MS operated in multiple reaction monitoring (MRM) mode. Transitions were as previously described [24, 25]; fumonisins B_2 and B_3 quantifier *m/z* 706→336, qualifier *m/z* 706→512 a; fumonisin B_4 quantifier *m/z* 690→320, qualifier *m/z* 690→514 a; fumonisins B_1 and B_6 quantifier *m/z* 722→334, qualifier *m/z* 722→528 a. Reference standards of FB_1–FB_4 and FB_6 were co-analyzed with each analysis sequence. Strains of *T. cylindrosporum* and *T. geodes* (CCF and DSM) were analyzed according to Vishwanath et al. [44] on a similar instrument.

LC–TOF-MS screening

Selected extracts were additionally tested for other fumonisins, efrapeptins, and cyclosporines by liquid chromatography time-of-flight mass spectrometry (LC–TOF-MS) as described in Nielsen et al. [28]. This was performed on an LC system coupled to an orthogonal TOF mass spectrometer (Micromass LCT, Manchester, UK) equipped with an electrospray source [28]. The column was a 50 × 2 mm i.d., 3 µm Luna C_{18} (II) column (Phenomenex, Torrance, California) and a linear gradient was used with all solutions containing 20 mM formic acid, from 15% acetonitrile in water to 100% acetonitrile in 20 min at 300 µL/min; this was maintained for 3.5 min before returning to the starting conditions in 6 min. The MS operated in ESI^+ at a scan range of *m/z* 100–2,000. Peaks not matching the compounds in an internal database with approximately 850 reference standards [29] were matched against the 35,500 structures in Antibase 2009 (John Wiley and Sons, Inc, Hoboken, New Jersey). Extracted ion chromatograms (±0.02 amu) of the $[M + H]^+$ ions of the fumonisin series A, C, or P were constructed to search specifically for these compounds.

Results and discussion

A screening for fumonisins found that all eleven *Tolypocladium* strains used in this study produced fumonisins B_2 (FB_2) and nine (82%) also produced fumonisins B_4 (FB_4); however, no FB_1, FB_3 or FB_6 was detected in any of the strains (representative chromatograms in Fig. 2). All *T. cylindrosporum* and *T. inflatum* strains and one *T. geodes* strain (CCF 2579) produced both FB_2 and FB_4, while the other two *T. geodes* strains produced only FB_2 (Table 1).

Three strains of *T. inflatum* (IBT 41581, IBT 41582, IBT 41583) were selected for quantitative analysis of fumonisin production on a variety of different media and at different conditions. We tested 10 different media, and found that on each medium at least one *T. inflatum* strain produced FB_2

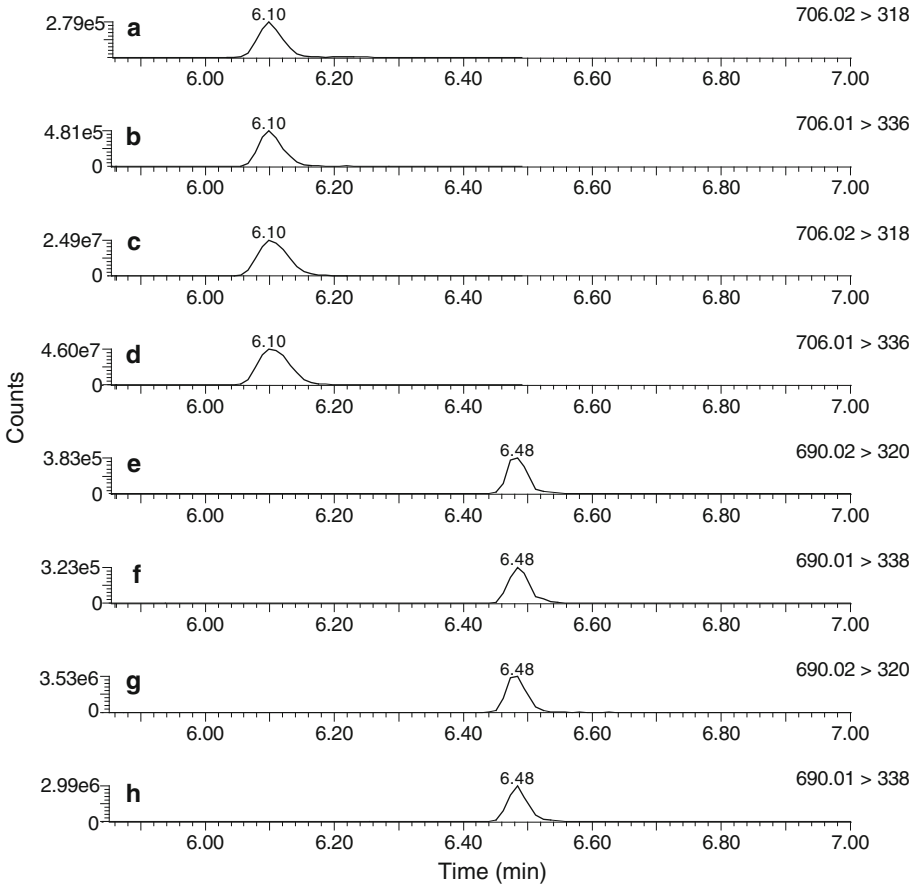

Fig. 2 LC–MS/MS analyses of fumonisin B_2 and B_4 in *Aspergillus niger* IBT 28144 and *Tolypocladium inflatum* IBT 41581 extracts from cultures incubated at 25°C for 7 days on YES. **a** Qualifier of a diluted certified FB_2 standard. **b** Quantifier of a diluted certified FB_2 standard. **c** Qualifier of FB_2 in a *T. inflatum* extract. **d** Quantifier of FB_2 in a *T. inflatum* extract. **e** Qualifier of FB_4 in an *A. niger* extract. **f** Quantifier of FB_4 in an *A. niger* extract. **g** Qualifier of FB_4 in a *T. inflatum* extract. **h** Quantifier of FB_4 in a *T. inflatum* extract

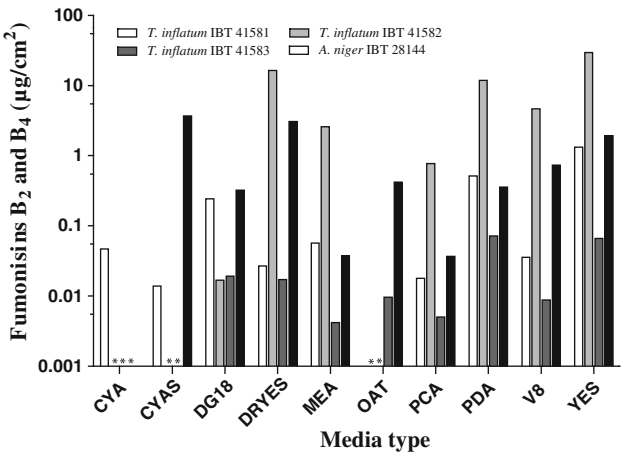

Fig. 3 Production of fumonisins B_2 and B_4 by three *Tolypocladium inflatum* strains (IBT 41581, IBT 41582, IBT 41583) and one *Aspergillus niger* (IBT 28144) on different media at 25°C. *Not detected

and FB_4 (Fig. 3). The maximal production of total fumonisins for the three strains was 1.3 µg/cm^2 for IBT 41581, 30 µg/cm^2 for IBT 41582 and 0.072 µg/cm^2 for IBT 41583. These levels were comparable to both *Fusarium* spp. and *A. niger* in a similar study under similar conditions, which found fumonisin production of 0.006–22 µg/cm^2 for *Fusarium* spp. and 0.27–21 µg/cm^2 for *A. niger* [26]. Relative levels of FB_4 to FB_2 were in the range 0–95%, with most being in the 10–30% range. The media yielding the highest amounts of fumonisins were DRYES, PDA, and YES, while lower amounts were detected in cultures grown on MEA, OAT, CYA, CYAS, PCA, V8, and DG18. *Aspergillus niger* (IBT 28144) produced high amounts of fumonisins on media with high amounts of sugar and salt, e.g., CYAS, DRYES, and YES, with detectable production of fumonisins on the other seven media as shown in Fig. 3. This is in agreement with the results of Frisvad et al. [13]. Fumonisin production by the *T. inflatum* strains was similar to that by both *Fusarium* and *A. niger*, with PDA and YES also supporting substantial fumonisin production [13, 26]. The media used for cyclosporine production often consists of 2–8% carbon source (e.g., sorbose, glucose, or similar) and 1–6% nitrogen source (e.g., peptone, malt extract, or casein acid hydrolysate) with added trace metals [2, 3, 17]. The media most comparable to these in our study are CYA, CYAS, and MEA. On CYA and CYAS, only one strain produced fumonisin, whereas all three strains produced fumonisins on MEA. This suggests that the media used for cyclosporine production might support the simultaneous

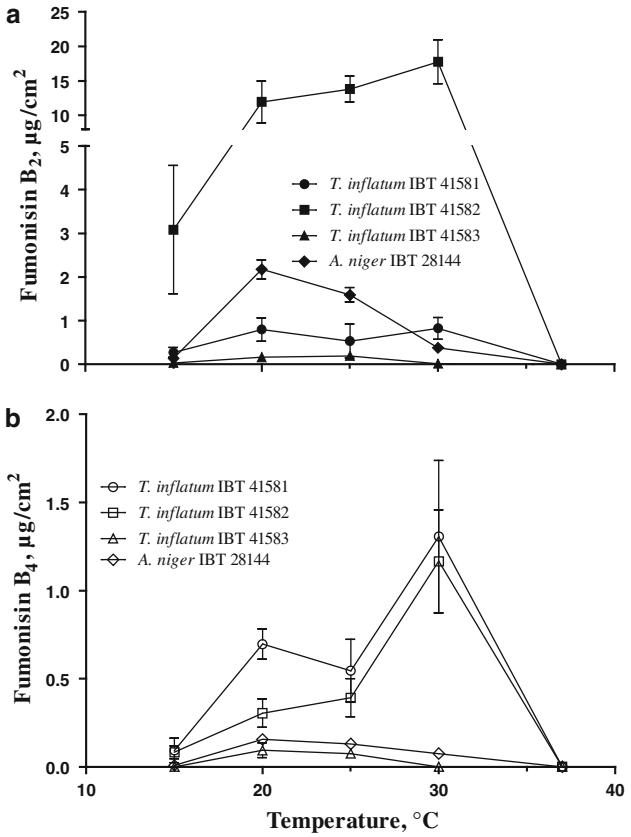

Fig. 4 Production of fumonisins B_2 (**a**) and B_4 (**b**) by three strains of *Tolypocladium inflatum* (IBT 41581, IBT 41582 and IBT 41583) and *Aspergillus niger* IBT 28144 incubated for 7 days at 15, 20, 25, 30, and 37°C on YES. The standard deviation of triplicates is shown by the error bars. Fumonisin concentration was 0 µg/cm^2 at 37°C because no growth occurred at that temperature

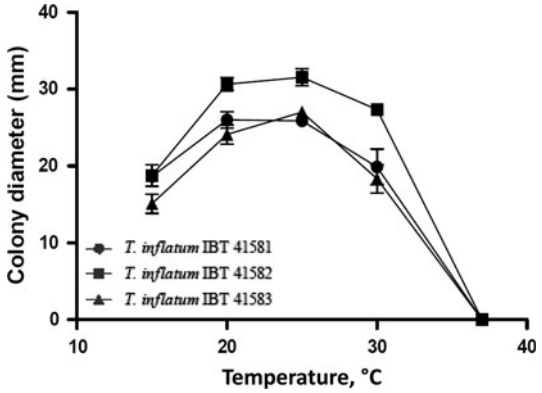

Fig. 5 Colony diameters of three strains of *Tolypocladium inflatum* (IBT 41581, IBT 41582, IBT 41583) after incubation for 7 days at 15, 20, 25, 30, and 37°C on YES. The standard deviation of triplicates is shown by the error bars

production of fumonisins, although the use of liquid medium compared to solid has to be accounted for.

The three *T. inflatum* strains produced fumonisins in a temperature range of 15–30°C (Fig. 4). The effect of temperature on fumonisin production was strain-dependent, with two strains giving maximal production at 30°C and one strain (IBT 41583) showing minimal production at 30°C, and maximum production at 25°C. This is similar to previous observations for *A. niger*, which showed peak fumonisin production at 25–30°C [26]. Except for one set of experiments in which IBT 41582 produced more FB_4 than FB_2 at 30°C, FB_2 was consistently produced at higher levels than FB_4 (data not shown). All three strains of *T. inflatum* were able to grow at temperatures from 15 to 30°C, with maximal colony diameters after 7 days of incubation at 20 or 25°C (Fig. 5). No growth was observed at 37°C.

In the three selected *T. inflatum* strains, no traces of fumonisin series A, C, or P were detected by LC–TOF-MS. However, FB_2 and FB_4 were detected in all samples of all cultures of the three *T. inflatum* strains in repeated experiments, and by the two different techniques of LC–MS/MS and LC–TOF-MS. Fumonisins were detected from cultures grown on different media (PDA, DRYES, and YES), and in separate cultures derived from the same original strain. In addition to FB_2 and FB_4, the three *T. inflatum* strains produced cyclosporines and efrapeptins with the fumonisins (data not shown).

This is the first report describing the production of FB_2 and FB_4 in the genus *Tolypocladium*. These findings are relevant because *T. inflatum* is used in the pharmaceutical industry as a producer of cyclosporin A. We recommend that the production strains of cyclosporines are analyzed for production of fumonisins under industrial production conditions to determine if these mycotoxins end up in the final product. Furthermore *Tolypocladium* spp. have been suggested as a possible fungal biological control agent, because of the entomopathogenicity of its production of efrapeptins [40]. With the discovery of fumonisin production by *Tolypocladium* spp., however, this raises concerns that these toxic metabolites may enter the environment and the food chain and pose a risk to humans and animals.

To date more than 20 species within the three genera *Fusarium*, *Aspergillus,* and *Tolypocladium* have been shown to produce fumonisins [13, 33]. *Alternaria arborescens* (as *Alternaria alternata* f. sp. *lycopersici*) was reported to produce fumonisins, but the results were later questioned and found to be due to only the chemically similar AAL toxin and not fumonisin [8, 9, 38]. So far, *Fusarium* spp. produce the greatest diversity of fumonisins, namely FB_1–FB_5 and the A, C, and P series [5, 7, 27], whereas *A. niger* produces only FB_2, FB_4, and FB_6 [13, 20, 30]. The discovery reported here shows that *Tolypocladium* spp. have a fumonisin profile similar to *A. niger*, but quite different from *Fusarium* spp. Because of these similar profiles, *Tolypocladium* spp. and *A. niger* could have a

higher degree of similarity in their fumonisin gene cluster compared to *Fusarium* spp., but this needs to be established by genome sequencing of the strains. This may help to determine if the genes were horizontally transferred and, if so, between which species. The discovery of fumonisin production in three distantly related fungal genera shows that the production of fumonisins may be more widespread in the fungal kingdom than previously believed.

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References

1. Baker SE (2006) *Aspergillus niger* genomics: past, present and into the future. Med Mycol 44:S17–S21
2. Balakrishnan K, Pandey A (1996) Influence of amino acids on the biosynthesis of cyclosporin A by *Tolypocladium inflatum*. Appl Microbiol Biotech 45:800–803
3. Balaraman K, Mathew N (2006) Optimization of media composition for the production of cyclosporin A by *Tolypocladium* species. Indian J Med Res 123:525–530
4. Bandani AR, Khambay BPS, Faull JL, Newton R, Deadman M, Butt TM (2000) Production of efrapeptins by *Tolypocladium* species and evaluation of their insecticidal and antimicrobial properties. Mycol Res 104:537–544
5. Bezuidenhout SC, Gelderblom WCA, Gorst-Allman CP, Horak RM, Marasas WFO, Spiteller G, Vleggaar R (1988) Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. J Chem Soc Chem Commun 11:743–745
6. Bissett J (1983) Notes on *Tolypocladium* and related genera. Can J Bot 61:1311–1329
7. Branham BE, Plattner RD (1993) Isolation and characterization of a new fumonisin from liquid cultures of *Fusarium moniliforme*. J Nat Prod 56:1630–1633
8. Caldas ED, Jones AD, Ward B, Winter CK, Gilchrist DG (1994) Structural characterization of 3 new AAL toxins produced by *Alternaria alternata* f. sp. *lycopersici*. J Agric Food Chem 42:327–333
9. Chen JP, Mirocha CJ, Xie WP, Hogge L, Olson D (1992) Production of the mycotoxin fumonisin B_1 by *Alternaria alternata* f. sp. *lycopersici*. Appl Environ Microbiol 58:3928–3931
10. EEC (2007) Setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. Comm Eur Communities 1126:14–16
11. Frisvad JC (1983) A selective and indicative medium for groups of *Penicillium viridicatum* producing different mycotoxins in cereals. J Appl Bacteriol 54:409–416
12. Frisvad JC, Samson RA (2004) Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*—A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. Stud Mycol 49:1–173
13. Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U (2007) Fumonisin B_2 production by *Aspergillus niger*. J Agric Food Chem 55:9727–9732
14. Gelderblom WCA, Jaskiewicz K, Marasas WFO, Thiel PG, Horak RM, Vleggaar R, Kriek NPJ (1988) Fumonisins-Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. Appl Environ Microbiol 54:1806–1811
15. Hocking AD, Pitt JI (1980) Dichloran glycerol medium for enumeration of xerophilic fungi from low moisture foods. Appl Environ Microbiol 39:488–492
16. Hodge KT, Krasnoff SB, Humber RA (1996) *Tolypocladium inflatum* is the anamorph of *Cordyceps subsessilis*. Mycologia 88:715–719
17. Isaac CE, Jones A, Pickard MA (1990) Production of cyclosporins by *Tolypocladium niveum* strains. Antimicrob Agents Chemother 34:121–127
18. Krasnoff SB, Gupta S, Stleger RJ, Renwick JAA, Roberts DW (1991) Antifungal and insecticidal properties of the efrapeptins—metabolites of the fungus *Tolypocladium niveum*. J Invertebr Pathol 58:180–188
19. Logrieco A, Doko B, Moretti A, Frisullo S, Visconti A (1998) Occurrence of fumonisin B_1 and B_2 in *Fusarium proliferatum* infected asparagus plants. J Agric Food Chem 46:5201–5204
20. Månsson M, Klejnstrup ML, Phipps RK, Nielsen KF, Frisvad JC, Gotfredsen CH, Larsen TO (2010) Isolation and NMR characterization of fumonisin B_2 and a new fumonisin B_6 from *Aspergillus niger*. J Agric Food Chem 58:949–953
21. Marasas WFO, Riley RT, Hendricks KA, Stevens VL, Sadler TW, Gelineau-van Waes J, Missmer SA, Cabrera J, Torres O, Gelderblom WCA, Allegood J, Martinez C, Maddox J, Miller JD, Starr L, Sullards MC, Roman AV, Voss KA, Wang E, Merrill Jr AH (2004) Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. J Nutr 134:711–716
22. Marin S, Ramos AJ, Vazquez C, Sanchis V (2007) Contamination of pine nuts by fumonisin produced by strains of *Fusarium proliferatum* isolated from *Pinus pinea*. Lett Appl Microbiol 44:68–72
23. Martins ML, Martins HM, Bernardo F (2001) Fumonisins B_1 and B_2 in black tea and medicinal plants. J Food Prot 64:1268–1270
24. Mogensen JM, Frisvad JC, Thrane U, Nielsen KF (2010) Production of fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. J Agric Food Chem 58:954–958
25. Mogensen JM, Larsen TO, Nielsen KF (2010) Widespread occurrence of the mycotoxin fumonisin B_2 in wine. J Agric Food Chem 58:4853–4857
26. Mogensen JM, Nielsen KF, Samson RA, Frisvad JC, Thrane U (2009) Effect of temperature and water activity on the production of fumonisins by *Aspergillus niger* and different *Fusarium* species. BMC Microbiol 9:281
27. Musser SM, Gay ML, Mazzola EP, Plattner RD (1996) Identification of a new series of fumonisins containing 3-hydroxypyridine. J Nat Prod 59:970–972
28. Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC (2009) Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Anal Bioanal Chem 395:1225–1242
29. Nielsen KF, Smedsgaard J (2003) Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for dereplication by standardised liquid chromatography-UV-mass spectrometry methodology. J Chromatogr A 1002:111–136
30. Noonim P, Mahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA (2009) Fumonisin B_2 production by *Aspergillus niger* from Thai coffee beans. Food Addit Contam 26:94–100
31. Park JW, Choi SY, Hwang HJ, Kim YB (2005) Fungal mycoflora and mycotoxins in Korean polished rice destined for humans. Int J Food Microbiol 103:305–314

32. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JAE, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EGJ, Debets AJM, Dekker P, van Dijck PWM, Van Dijk A, Dijkhuizen L, Driessen AJM, d'Enfert C, Geysens S, Goosen C, Groot GSP, de Groot PWJ, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JPTW, van den Hondel CAMJ, van der Heijden RTJM, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, LU X, van der Maarel MJEC, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NNME, Ram AFJ, Rinas U, Roubos JA, Sagt CMJ, Schmoll M, Sun JB, Ussery D, Varga J, Vervecken W, de Vondervoort PJJV, Wedler H, Wösten HAB, Zeng AP, van Ooyen AJJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 25:221–231
33. Rheeder JP, Marasas WFO, Vismer HF (2002) Production of fumonisin analogs by *Fusarium* species. Appl Environ Microbiol 68:2101–2105
34. Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B (2010) Food and indoor fungi. CBS-KNAW Fungal Biodiversity Centre, Utrecht
35. Samson RA, Soares GG (1984) Entomopathogenic species of the hyphomycete genus *Tolypocladium*. J Invertebr Pathol 43:133–139
36. Scott PM, Lawrence GA (1995) Analysis of beer for fumonisins. J Food Prot 58:1379–1382
37. Simmons EG (2007) *Alternaria.* An identification manual. CBS-KNAW Fungal Biodiversity Centre, Utrecht
38. Solfrizzo M, de Girolamo A, Vitti C, Tylkowska K, Grabarkiewicz-Szczesna J, Szopinska D, Dorna H (2005) Toxigenic profile of *Alternaria alternata* and *Alternaria radicina* occurring on umbelliferous plants. Food Addit Contam 22:302–308
39. Stack ME (1998) Analysis of fumonisin B_1 and its hydrolysis product in tortillas. J AOAC Int 81:737–740
40. Strasser H, Vey A, Butt TM (2000) Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium, Tolypocladium* and *Beauveria* species? Biocontrol Sci Tech 10:717–735
41. Sung G-H, Hywel-Jones NL, Sung J-M, Luangsa-ard JJ, Shrestha B, Spatafora JW (2007) Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. Stud Mycol 57:5–59
42. Sydenham EW, Shephard GS, Thiel PG, Marasas WFO, Stockenstrom S (1991) Fumonisin contamination of commercial corn-based human foodstuffs. J Agric Food Chem 39:2014–2018
43. US Food and Drug Administration (2001) Guidance for industry: fumonisin levels in human foods and animal feeds. United States Food and Drug Administration, Washington, DC
44. Vishwanath V, Sulyok M, Labuda R, Bicker W, Krska R (2009) Simultaneous determination of 186 fungal and bacterial metabolites in indoor matrices by liquid chromatography/tandem mass spectrometry. Anal Bioanal Chem 395:1355–1372
45. Voss KA, Riley RT, Snook ME, Gelineau-van Waes J (2009) Reproductive and sphingolipid metabolic effects of fumonisin B_1 and its alkaline hydrolysis product in LM/Bc mice: hydrolyzed fumonisin B_1 did not cause neural tube defects. Toxicol Sci 112:459–467
46. Wild CP, Gong YY (2010) Mycotoxins and human disease: a largely ignored global health issue. Carcinogenesis 31:71–82
47. Zhu XC, Vogeler C, Du LC (2008) Functional complementation of fumonisin biosynthesis in *FUM*1-disrupted *Fusarium verticillioides* by the AAL-toxin polyketide synthase gene *ALT*1 from *Alternaria alternata* f. sp. *lycopersici*. J Nat Prod 71:957–960

Supplementary Table 1: Media composition

Media	Content
CYA	Agar (15 g/l), Yeast extract (5 g/l) (Biokar Diagnostics), Czapek Dox Broth [Saccharose (30 g/l), $NaNO_3$ (3 g/l), K_2PO_4 (1 g/l), TMS (1 ml/l), $MgSO_4$(50 mg/l), KCl (50 mg/l), $FeSO_4$ (0.01mg/l)] (DIFCO)
CYAS	NaCl (5 g/l), Agar (15 g/l), Yeast extract (5 g/l) (Biokar Diagnostics), Czapek Dox Broth [Saccharose (30 g/l), $NaNO_3$ (3 g/l), K_2PO_4 (1 g/l), TMS (1 ml/l), $MgSO_4$ (50 mg/l), KCl (50 mg/l), $FeSO_4$ (0.01 mg/l)] (DIFCO)
DG18	Glycerol (180 g/l), TMS (0.8 ml/l), Chloramphenicol (0.1 g/l), Dichloran-Glycerol agar base [Glucose (10 g/l), Bacto-Peptone (5 g/l), $Na_2H_2PO_4$ (1 g/l), $MgSO_4$ (0. 5 g/l), Dichloran (0.2 mg/l)] (OXOID)
DRYES	Sucrose (150 g/l), Yeast extract (20 g/l) (Biokar Diagnostics), Agar (20 g/l), TMS (1 ml/l), $MgSO_4$ (0.5 g/l), Chloramphenicol (0.1 g/l), Rose Bengal (5 mg/l), Dichloran (0.2 mg/l)
MEA	Malt extract (20 g/l) (DIFCO), Glucose (20 g/l), Agar (20 g/l), Peptone (1 g/l), TMS (1 ml/l)
OAT	Oat meal (30 g/l), Agar (15 g/l), TMS (1 ml/l)
PCA	Potatoes (10 g/l), Carrots (10 g/l), TMS (0.5 ml/l), Agar (10 g/l)
PDA	Dextrose (20 g/l), Agar (15 g/l), Potato starch (4 g/l), TMS (1 ml/l)
V8	Campbell's V8 Juice (175 g/l), $CaCO_3$ (3 g/l)
YES	Sucrose (150 g/l), Yeast extract (20 g/l), Agar (20 g/l), TMS (1 ml/l), $MgSO_4$ (0.5 g/l),

TMS: Trace metals solution (Stock solution, $ZnSO_4 \cdot 7H_2O$ (10 g/l), $CuSO_4 \cdot 5H_2O$ (5 g/l))

Paper 9

”Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group”

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REVIEW

Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group

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Abstract Filamentous fungi in the *Aspergillus* section *Nigri* (the black aspergilli) represent some of the most widespread food and feed contaminants known but they are also some of the most important workhorses used by the biotechnological industry. The *Nigri* section consists of six commonly found species (excluding *A. aculeatus* and its close relatives) from which currently 145 different secondary metabolites have been isolated and/or detected. From a human and animal safety point of view, the mycotoxins ochratoxin A (from *A. carbonarius* and less frequently *A. niger*) and fumonisin B_2 (from *A. niger*) are currently the most problematic compounds. Especially in foods and feeds such as coffee, nuts, dried fruits, and grape-based products where fumonisin-producing fusaria are not a problem, fumonisins pose a risk. Moreover, compounds such as malformins, naptho-γ-pyrones, and bicoumarins (kotanins) call for monitoring in food, feed, and biotechnology products as well as for a better toxicological evaluation, since they are often produced in large amounts by the black aspergilli. For chemical differentiation/identification of the less toxic species the diketopiperazine asperazine can be used as a positive marker since it is consistently produced by *A. tubingensis* (177 of 177 strains tested) and *A. acidus* (47 of 47 strains tested) but never by *A. niger* (140 strains tested). Naptho-γ-pyrones are the compounds produced in the highest quantities and are produced by all six common species in the group (*A. niger* 134 of 140; *A. tubingensis* 169 of 177; *A. acidus* 44 of 47; *A. carbonarius* 40 of 40, *A. brasiliensis* 18 of 18; and *A. ibericus* three of three).

Keywords Metabolomics · Fumonisin · Ochratoxin · Liquid chromatography–mass spectrometry · Polyketide synthase · Polyketide

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Introduction

The black aspergilli are some of the most important mycotoxigenic food and feed contaminants, especially in postharvest decay of fresh and dried fruits and certain vegetables, nuts, beans, and cereals [1, 2]. This is due to their fast growth, pH tolerance, and high abundance in many environments.

For the analytical chemist, issues such as fungal taxonomy and correct identification may seem of low relevance, but in fact biosystematics is a vital part of mycotoxin research and food safety. Since the profile of mycotoxins and other secondary metabolites is species-specific [3–5], correct identification at the species level provides the key for planning the analytical determination of all relevant compounds.

The *Aspergillus niger* group (the black aspergilli, *Aspergillus* subgenus *Circumdati* section *Nigri*) comprises 18 species, of which *A. niger, A. tubingensis, A. brasiliensis, A. acidus, A. carbonarius*, and *A. ibericus* are common, whereas the remaining species are rare and found mainly in tropical regions (Table 1). A cladification of *Aspergillus* section *Nigri* using the β-tubulin and calmodulin genes showed that three clades could be distinguished [5]: the *A. niger* clade, a clade consisting of the two rare species *A. homomorphus* and *A. ellipticus,* and the clade of uniseriate black aspergilli (*A. aculeatinus, A. aculeatus, A. japonicus*,

Table 1 Species in the *Aspergillus niger* group

Species	Rare	Species	Rare
A. acidus[a]		*A. ibericus*	
A. brasiliensis[a]		*A. niger*	
A. carbonarius		***A. piperis***	Yes
A. costaricaensis	Yes	***A. sclerotiicarbonarius***	Yes
A. ellipticus	Yes	***A. sclerotioniger***	Yes
A. heteromorphus	Yes	*A. tubingensis*[a]	
A. homomorphus	Yes	*A. vadensis*	Yes
Uniseriate black aspergilli			
A. aculeatinus		***A. japonicus***	
A. uvarum		***A. aculeatus***	

Species whose names are in **bold** have only been found in the tropics as yet. Important synonyms of *A. niger* are *A. awamori*, *A. phoenicis*, *A. kawachii*, *A. saitoi*, *A. usamii*, *A. foetidus*, *A. citricus*, and *A. ficuum*.

[a] Regularly confused with *A. niger*

and *A. uvarum*), the members of which differ significantly from the remaining black aspergilli regarding their morphology, physiological behavior, and secondary metabolite production (e.g., producers of neoxaline, asperparalines, secalonic acids, asperamide, and aculeasins) [5], and this third clade has therefore not been included in this review. The identity and metabolite production of the uniseriate black aspergilli is usually not confused with the identity and metabolite production of *A. niger* and other biseriate black aspergilli.

A. niger and *A. tubingensis* are probably the most common of the black aspergilli; however, in many studies describing secondary metabolites from these aspergilli, the producing organism has been identified as a black *Aspergillus* and then in many cases incorrectly named *A. niger*. A wrong identification may be further complicated by insufficient molecular identification based on sequencing of ribosomal DNA with low resolution [5, 6]. A polyphasic approach where many different types of characters (microscopy, metabolite profiling, molecular methods) are used is recommended for the identification of these aspergilli. Certain molecular methods have proven quite successful, including restriction fragment length polymorphism and β-tubulin, or calmodulin sequencing (reviewed in [5]).

Until recently, the main mycotoxin from the black aspergilli was considered to be ochratoxin A (OTA), produced in variable amounts within certain species of the group. *A. carbonarius* consistently produces large amounts of OTA, whereas only 6–10% of members of the *A. niger* group produce OTA and in 10–1000 lower amounts [7–9]. The third species producing OTA in section *Nigri* is *A. sclerotioniger*, but this species has only been found once in coffee. However, *A. niger* clearly has the widest distribution and has been reported to grow and damage a much larger number of crops and foods worldwide, including corn, peanuts, raisins, onions, mango, apples, and dried meat products [2]. This combined with the recent discovery that *A. niger* can also produce fumonisin B_2 (FB_2) and fumonisin B_4 (FB_4) [10, 11] (Fig. 1) necessitates the addition of fumonisins in a number of food and feed screening programs.

A. niger exhibits a remarkably versatile metabolism, which has made it one of the most important production organisms used for industrial fermentations [12, 13]. Since 1923, *A. niger* has been exploited commercially for its production of citric acid, mostly for use in food, cosmetics, and pharmaceutical preparations [14]. In addition, the fungus has been a rich source of industrial enzymes such as α-amylases, cellulases, and pectinases for use in the food industries since the 1960s [12]. *A. niger* possesses posttranslational mechanisms capable of correctly processing proteins that are difficult to express in traditional host organisms. As a result, it is widely used as a cell factory for heterologous expression of proteins [14].

A. niger has been considered to be nontoxic under industrial conditions [12], and thus to be considered a safe production organism. As a result, quite a number of *A. niger* fermentations have been granted the generally regarded as safe (GRAS) status by the US Food and Drug Administration [12]. However, the potential presence of both OTA and fumonisins in *A. niger* emphasizes the need to adjust and/or reconsider the screening procedures for simultaneous targeting of multiple classes of mycotoxins.

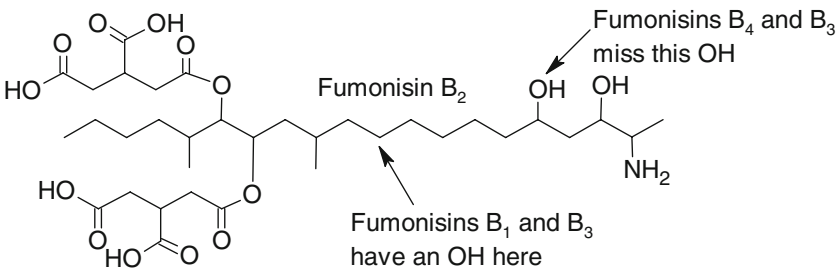

Fig. 1 Structure of fumonisin B_2 and the difference from the structures of fumonisin B_1, fumonisin B_3, and fumonisin B_4

In this review, we focus on the important secondary metabolites produced by members of the *A. niger* group relevant to the food, feed, and biotechnology industries. We have critically scrutinized the existing literature for reports of secondary metabolites claimed to be produced by *A. niger*, however often just found in a single strain or on a single occasion. In addition, we present analytical results from 25 years of metabolite profiling at the Center for Microbial Biotechnology of the black aspergilli. The strains investigated come from a large in-house collection (IBT collection, author address) and other fungal collections. The results are based on continuously obtained data from liquid chromatography (LC) with diode-array detection (DAD), 6 years of LC-DAD analyses combined with high-resolution time-of-flight (TOF) mass-spectrometric detection, and 1 year of screening with LC and tandem mass-spectrometric detection.

The overall aim is to provide an overview of the large numbers of compounds produced within this important group of filamentous fungi and to identify potential difficulties and pitfalls in the biochemical analysis of these compounds. Therefore, we discuss the individual compound classes, their biological significance, and natural abundance together with their spectroscopic and chromatographic properties relevant for their analytical determination.

Methodologies applied at the center for microbial biotechnology

Cultivation and extraction

Data obtained from analysis of *A. niger* group strains in our institution during the last 25 years were compiled. Cultures were identified on the basis of their morphology, metabolite profile, and partial sequencing (β-tubulin and calmodulin) as described elsewhere [15]. The strains were grown and extracted by one of the following three methods:

1. Combined chloroform–methanol (2:1, v/v) and acetone–ethyl acetate (1:1, v/v) extracts from cultures on yeast extract sucrose (YES) agar, Sigma YES agar, oatmeal agar, and potato sucrose agar (three plates each), as described by Frisvad and Thrane [16].These were made in the years 1983–1995, and comprise approximately 150 fungal isolates.
2. Extracts made by the microextraction procedure of Smedsgaard [17], where approximately 0.6-cm^2 plugs of culture from YES agar, Czapek yeast autolysate agar (CYA), CYA with 5% salt (CYAS), oatmeal agar, or malt extraxt agar [4] were extracted with methanol–dichloromethane–ethyl acetate (1:2:3 v/v) and 1% formic acid. These comprise some 200 fungal isolates and 200 extracts made in the years 1996–2009.
3. Extracts for fumonisin analysis made by the microextraction procedure mentioned above, but extracted using 75% methanol [10] in the years 2007–2009. Here mainly CYAS and partly YES agar cultures were extracted, which add up to a further 100 cultures and 200 extracts.

Metabolite analysis by LC-DAD

The large extracts from the years 1983–1995 were analyzed by LC [acidic, 15–100% acetonitrile (CH_3CN) gradient, 40 min, Nucleosil C_{18} column] with DAD (200–600 nm) as described by Frisvad and Thrane [16]. Data were available as printed reports with chromatographic traces (210 and 280 nm) and UV/vis spectra (200–600 nm).

Microextracts from the years 1995–2003 were analyzed by LC-DAD with parallel fluorescence detection (FLD) under similar chromatographic conditions using a Nucleosil BDB C_{18} column [17] with the FLD set at 230→333 and 230→450 nm.

Microextracts from the years 2003–2009 were analyzed by LC-DAD-FLD as mentioned above using a Luna C_{18} II column (15% CH_3CN to100% CH_3CN in 20 min) [18].

Metabolite analysis by LC-DAD–TOF mass spectrometry

These analyses were performed using a LC system coupled to an orthogonal TOF mass spectrometer (Micromass LCT oaTOF) equipped with an electrospray source [19]. Two different gradients were used: (1) Luna II C_{18} 15% CH_3CN to 100% CH_3CN in 20 min, 20 mM formic acid in both solvents [20]; (2) as described before but with the gradient starting at 30% CH_3CN and going to 60% CH_3CN in 5 min and then to 100% in 1 min.

About 300 extracts were analyzed by LC-DAD–TOF mass spectrometry (TOFMS) in the years 2003–2009; of these, most have also been analyzed by LC-DAD-FLD.

Metabolite analysis by LC–tandem mass spectrometry

This was performed using a Quattro Ultima triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source and operated in multiple reaction mode (MRM) [11]. Separation was done on a Gemini C_6-phenyl column (acidic, 20% CH_3CN to 55% CH_3CN in 6 min, then to 100% in 30 s). MRM transitions for OTA, fumonisin B_1 (FB_1), FB_2, fumonisin B_3 (FB_3), and FB_4 are described elsewhere [11, 21]. Malformin A_2 was detected using *m*/*z* 516→304 at 30 V and *m*/*z* 516→417 at 20 V; malformins C and A were detected using *m*/*z* 530→372 at 25 V and *m*/*z* 530→417 at 20 V; ochratoxin β was detected using *m*/*z* 223→103 at 36 V and *m*/*z* 223→159 at 36 V; ochratoxin α was detected using *m*/*z* 257→193 at 36 V and

m/z 257→221 at 33 V; and ochratoxin B was detected using *m/z* 370→205 at 36 V and *m/z* 370→324 at 36 V. About 350 extracts were analyzed by LC–tandem mass spectrometry (MS/MS) in the years 2008–2009; many of these have also been analyzed by LC-TOFMS and LC-DAD-FLD.

Reference standards

For the LC-DAD-FLD analyses, OTA, ochratoxin B (Sigma-Aldrich), ochratoxin α, kotanin, nigragillin, and malformins A, A_2, and C (gifts) were coanalyzed. For LC–mass spectrometry (MS), FB_1, FB_2, and FB_3 (Biopure, Tuln, Austria) and AAL toxins TA_1 and TB_1 (Sigma-Aldrich) were also included. Tensidols A and B and aurasperones A, B, and E were purified and validated by NMR [22].

Data analysis

LC-DAD and LC-DAD-FLD data were analyzed manually by comparing retention times, and UV spectra for the detected peaks as well as the retention times of the fluorescence peaks. These data were matched with LC-DAD-TOFMS data for tentative identification of the peaks by searching Antibase (Wiley) for compounds with the same accurate mass (±0.01-Da tolerance) and similar UV properties and estimated retention time (based on theoretical log*D* values) [19]. Compounds that we were able to tentatively identify in extracts from black aspergilli were aurasperones C–G, pyrophen, funalenone, fonsecinones B and C, fonsecin (TMC-256B1B), TMC-256A2, rubrofusarin B, fonsecin B, tensyuic acid A, asperazine, pyranonigrins A–D, and FB_4. The identity of FB_4 was further validated by showing that its accurate mass, retention time and tandem spectra from 25–50 eV were identical to those of to the presumed FB_4 in culture extracts from *Fusarium verticillioides*.

Analysis of biological significant metabolites

The black aspergilli can produce a diverse range of mainly polyketide-derived secondary metabolites along with nonribosomal peptides and a number of compounds of mixed biosynthetic origin. Annotation of the fully sequenced genome of *A. niger* showed the presence of an impressive 34 polyketide synthase (PKS)-encoding genes as well as 17 nonribosomal peptide synthase (NRPS) genes and seven PKS-NRPS hybrids, accentuating the biosynthetic potential and versatility of this species [13]. So far, a total of 145 metabolites (Table 2) have been isolated and their structures elucidated from the biseriate black aspergilli.

Ochratoxins

Historically, OTA and its precursors (ochratoxins B, β, and α) have been the most significant mycotoxins produced within the *A. niger* complex. In extracts from approximately 400 agar cultures, OTA was always detected in amounts at least 10 times higher than the precursors using LC-FLD, LC-TOF analysis, and LC-MS/MS, and in many cases only OTA was detected.

It has been claimed that *A. tubingensis* can produce OTA [23, 24], presumably owing to unspecific chemical analyses. It was not possible to confirm this from the same cultures in our laboratory by LC-FLD, LC-TOF analysis, nor LC-MS/MS in culture extracts from up to ten different media. Other researchers have also doubted OTA production by *A. tubingensis* [3, 25].

Owing to the array of adverse toxicological effects on animals and humans, OTA is one of the most studied mycotoxins. The very low regulatory levels in food and feed mean that numerous highly sensitive analytical methods have been developed [26–28] and these have been reviewed several times [29, 30]. Thus, OTA will not be in the major scope of this paper since many of the commodities where *A. niger* is a problem have already been covered [29, 31–35]. However, it must be emphasized that we have found that direct analysis of OTA from *A. niger* using LC-DAD is not possible in crude extracts owing to coeluting malformins and naptho-γ-pyrones (NGPs) (see later), of which the NGPs are present in more than 100 times higher quantities than OTA. This is illustrated in Fig. 2 showing LC-DAD-TOFMS chromatogram with the OTA peak hidden underneath the peaks of rubrofusarin B and malformins. Furthermore, with use of our LC-DAD-FLD method, four to five peaks of closely eluted substances are detected from many black *Aspergillus* extracts (230→333 nm, acidic conditions). This indicates a risk that some of these compounds may be coeluted with OTA even under other slightly different conditions. Thus, careful control of chromatographic conditions is necessary. Careful control of potential carryover between samples in the autosampler should be done if high-level OTA-producing *A. carbonarius* strains are analyzed in sequence.

For OTA analysis, our LC-TOFMS was approximately twofold less sensitive than FLD. Here sensitivity could be enhanced tenfold by shifting to alkaline conditions [32]; however, other compounds would suffer from a pH change (e.g., by postcolumn addition of NH_4OH solution) and comparison with old data will not be possible. Our LC-MS/MS instrument is about 20–50 times more sensitive than acidic FLD and has until now not yielded any interfering peaks on any of the two MRM transitions. It should be noted that the latest LC-TOFMS and Orbitrap instruments will, according to the manufactures, provide 10 times

Table 2 Secondary metabolites and mycotoxins from *A. niger* and related species

Component	Species	Elementary composition	UV/VIS	Monoisotopic mass (Da)
Glyoxylic acid [86, 87]		$C_2H_2O_3$		74.0004
Glycolic acid [86]		C_2H_4O		76.0155
Oxalic acid [88]		$C_2H_2O_4$		89.9947
Hydroxypyruvic acid [89]		$C_3H_4O_4$		104.0104
(+)-Parasorbic acid (Antibase2008)		$C_6H_8O_2$		112.0519
Sorbic acid (Antibase2008)		$C_6H_8O_2$		112.0519
Fumaric acid [90]		$C_4H_4O_4$		116.0104
2-Phenylethanol [91]		$C_8H_{10}O$		122.0726
Glutaric acid [86]		$C_5H_8O_4$		132.0423
Asperyellone [92]		$C_{20}H_{22}O$		278.1671
Phenylacetic acid [91]		$C_8H_8O_3$		136.0519
Phenoxyacetic acid [91]		$C_8H_8O_3$		152.0468
p-Methoxyphenylacetic acid [91]		$C_9H_{10}O_3$		166.0624
4-Hydroxymandelic acid [88]		$C_8H_8O_4$		168.0417
Dehydrocarolic acid [93]	*A. brasiliensis*	$C_9H_8O_4$		180.0417
Cyclo-L-Ala-L-Leu [94]		$C_9H_{16}N_2O_2$		184.1206
Citric acid [95]	*Nigri*[a]	$C_6H_8O_7$		192.0265
D-Galactonic acid		$C_6H_{12}O_7$		196.0583
7-Hydroxy-4-methoxy-5-methylcoumarin [96, 97]	*A. niger* (reported as *Cladosporium herbarum*)	$C_{11}H_{10}O_4$		206.0574
Hexylitaconic acid [98, 99]	*A. niger*	$C_{11}H_{18}O_4$		214.1200
3-Methyl-8-hydroxy-4-decanoate [91]	*A. niger*	$C_{12}H_{22}O_3$		214.1564
Ochratoxin β	*A. niger, A carbonarius, A. sclerotioniger*	$C_{11}H_{10}O_5$		222.0523
Nigragillin [93]	*A. niger, A. tubingensis*	$C_{13}H_{22}ON_2$	262 (26,200)	222.1739
Pyranonigrin A (pyranopyrrol A) [57, 75]	*Nigri*	$C_{10}H_9NO_5$,	210, 252, 316	223.0481
Aspernigrin A [57, 96]	*A. niger*	$C_{13}H_{12}N_2O_2$		228.0893
Carbonarone A [83]	*A. carbonarius*	$C_{13}H_{11}NO_3$		229.0733
Carbonarone B [83]	*A. carbonarius*	$C_{13}H_{11}NO_3$		229.0733
Carbonarone A [83]	*A. carbonarius*	$C_{13}H_{11}NO_3$	279 (100%), 317 (29%), 401 (25%)	229.0739
Carbonarone B [83]	*A. carbonarius*	$C_{13}H_{11}NO_3$	340 (100%)	229.0739
Tensidol A [100]	*A. niger, A. tubingensis*	$C_{13}H_{11}NO_3$	206 (100%), 250 (30%)	229.0739
2-Methylene-3-hexyl-butanedioic acid [98]	*A. niger*	$C_{11}H_{18}O_5$		230.1149
Pyranonigrin D (pyranopyrrol D) [57]	*Nigri*	$C_{11}H_9NO_5$		235.0481
2-Carboxymethyl-3-hexylmaleic acid anhydride [98, 101]	*A. niger*	$C_{12}H_{16}O_5$	253	240.0992
Tensyuic acid [102]	*A. niger*	$C_{11}H_{16}O_6$	203 (6,000), 205 (5,600)	244.0947
Tensyuic acid F [102]	*A. niger*	$C_{11}H_{16}O_6$	199 (11,000)	244.0947
Pyranonigrin B (pyranopyrrol B) [57]	*Nigri*	$C_{11}H_{11}NO_6$		253.0586
Pyranonigrin C (pyranopyrrol C)[57]	*Nigri*	$C_{11}H_{11}NO_6$		253.0586
Ochratoxin α	*A. carbonarius, A. niger, A sclerotioniger*	C11H9ClO5		256.0133
Tensyuic acid B [102]	*A. niger*	$C_{12}H_{18}O_6$	204 (8,900), 207 (7,700)	258.1103
Nigerazine A [71]	*A. niger*?	$C_{16}H_{22}N_2O$		258.1727
Nigerazine B [71]	*A. niger*?	$C_{16}H_{22}N_2O$	280 (22,400)	258.1732
Nigerloxin [79]	*A. niger*?	$C_{13}H_{15}NO_5$		265.0945
TAFU-567 [103]	*A. acidus*?	$C_{13}H_{14}O_6$		266.0785
Antafumicin A [104]	*A. acidus*	$C_{13}H_{14}O_6$	217(21,000), 279(15,500), 317(8,720)	266.0786

Table 2 (continued)

Component	Species	Elementary composition	UV/VIS	Monoisotopic mass (Da)
Antafumicin B [104]	*A. acidus*	$C_{13}H_{14}O_6$	217(21,000), 279(15,500), 317(8,720)	266.0786
TMC-256A1 [105]	*A. niger*	$C_{15}H_{12}O_5$	236 (100%), 276 (68%), 368 (12%), 482 (1%)	272.0679
TMC-256C1 [105]	*A. niger*	$C_{15}H_{12}O_5$		272.0679
Rubrofusarin(heminigerone) [44, 106]	*Nigri*	$C_{15}H_{12}O_5$	(225, 28,200) (253sh, 19,600) (278, 49,700) (328, 3,400) (415, 5,200)	272.0679
Tensyuic acid C [102]	*A. niger*	$C_{13}H_{20}O_6$	201 (7,700), 206 (4,500)	272.1260
Tensyuic acid D [102]	*A. niger*	$C_{13}H_{20}O_6$	202 (17,000), 205 (7,700)	272.1260
Asperrubrol [64, 107]	*A. niger, A. tubingensis*	$C_{20}H_{22}O$	242(9,800), 293(11,200), 400(63,200)	278.1672
Asperic acid [65]	*A. niger*	$C_{16}H_{28}O_4$	225, 280	284.1988
Flavasperone [108, 109]	*Nigri*	$C_{16}H_{14}O_5$	225(28,184), 254(47,863), 406(5,495)	286.0841
Tensyuic acid E [102]	*A. niger*	$C_{14}H_{22}O_6$	198 (13,000)	286.1416
Pyrophen [65]	*A. tubingensis, A. niger*	$C_{16}H_{17}NO_4$		287.1158
Funalenone [55]	*A. niger, A. tubingensis, A brasiliensis*	$C_{15}H_{12}O_6$		288.0634
Fonsecin (TMC-256B1) [110]	*Nigri*	$C_{15}H_{14}O_6$		290.0790
Nigerasperone A [111]	*A. niger*?	$C_{16}H_{14}O_6$	210sh (55%), 226 (74%), 255sh (56%), 276 (100%), 325 (14%), 400 (22%)	302.0785
Xanthoherquein [55]	*A. sclerotioniger*?	$C_{15}H_{12}O_7$		304.0578
Fonsecin B (TMC-256B2) [105, 110, 112]	*Nigri*	$C_{16}H_{16}O_6$	231 (80%), 275 (100%), 328 (26%), 406 (21%)	304.0941
Fonsecin monomethyl ether [44, 110, 112]	*Nigri*	$C_{16}H_{16}O_6$	232(28,500), 277(40,500), 317(9,100), 330(10,000), 395(8,400)	304.0947
Asnipyrone B [113]	*A. niger*?	$C_{20}H_{20}O_3$	235(16,600), 265(15,490), 376(33,885)	308.1413
Carbonarin E [114]	*A carbonarius*	$C_{18}H_{17}NO_4$		311.1158
Asperenone [107]	*A. niger, A. tubingensis*	$C_{22}H_{24}O_3$	242(13,800), 300(9,200), 414–416(94,200)	312.1726
4,9-Dihydroxyperylene-3,10-quinone [88]	*Nigri*?	$C_{20}H_{10}O_4$		314.0573
Asnipyrone A [113]	*A. niger*	$C_{21}H_{22}O_3$	240(18,600), 290(19,055), 378(31,625)	322.1570
Atromentin [75]	*A. niger, A. tubingensis*	$C_{18}H_{12}O_6$		324.0628
Cycloleucomelon [57]	*A. niger, A. tubingensis*	$C_{18}H_{10}O_7$		338.0421
Tensidol B [100]	*A. niger, A. tubingensis*	$C_{18}H_{17}NO_6$	206 (100%),	343.1050
Nafuredin [115]	*A. niger*?	$C_{22}H_{32}O_4$		360.2295
22-Deacetylyanuthone A [77]	*A. niger, A. tubingensis*	$C_{22}H_{32}O_4$	238(16,220), 302(480)	360.2302
Aspergillin [54]	*A. Niger*	$C_{19}H_{12}O_8$		368.0527
2,3,4,8,9,10-Hexahydroxy-5*H*,6*H*,7*H*-benzopyrene-1,11-dione [88]	*Nigri*	$C_{19}H_{12}O_8$		368.0527
1-Hydroxyyanuthone A [77]	*A. niger, A. tubingensis*	$C_{24}H_{32}O_5$	234(8,510), 290(220)	400.2251
1-Hydroxyyanuthone C [77]	*A. niger, A. tubingensis*	$C_{24}H_{32}O_5$	232(4,680), 282(468)	400.2251
Yanuthone B [77]	*A. niger, A. tubingensis*	$C_{24}H_{32}O_5$	244(4,470), 290(1,950), 338(830)	400.2251
Tubingensin A [116]	*A. tubingensis* ATCC 76608[b]	$C_{28}H_{35}NO$		401.2713
Tubingensin B [117]	*A. tubingensis* ATCC 76608	$C_{28}H_{35}NO$	218(17,200), 237(25,500), 260(10,100), 299(10,100), 325(2,200), 338(6,700)	401.2720
Yanuthone A [77]	*A. niger, A. tubingensis*	$C_{24}H_{34}O_5$	232(7,950), 310(500)	402.2407
Yanuthone C [77]	*A. niger, A. tubingensis*	$C_{24}H_{34}O_5$	232(13,180), 280(2,040), 322(1,070)	402.2407
Ochratoxin A [118]	*A. niger, A. carbonarius, A. sclerotioniger*	$C_{20}H_{18}ClNO_6$	205(94%), 215(98%), 218(21%), 283(2%), 332(17%)	403.0823
Dihydrotubingensin B [119]	*A. tubingensis* ATCC 76608	$C_{28}H_{37}NO$		403.2870
Dihydrotubingensin A [119]	*A. tubingensis* ATCC 76608	$C_{28}H_{37}NO$		403.2870
10,23-Dihydro-24,25-dehydroaflavinine [120]	*A. tubingensis* ATCC 76608	$C_{28}H_{39}NO$	226(31,600), 284(4,400), 291(4,100)	405.3032
Aflavinine [120]	*A. tubingensis* ATCC 76608	$C_{28}H_{39}NO$	225(18,700), 283(3,010), 291(2,710)	405.3032
Bicoumanigrin (JH 0508 O'; bicumarine) [57]	*A. niger*	$C_{22}H_{18}O_8$		410.0996

Table 2 (continued)

Component	Species	Elementary composition	UV/VIS	Monoisotopic mass (Da)
Orlandin [56]	*A. niger*	$C_{22}H_{18}O_8$	311(29,500), 321(25,120)	410.1002
BMS-192548 [52, 121]	*A. niger*?	$C_{21}H_{18}O_9$	280(4,440), 320(35,548), 414(38,822)	414.0950
Nygerone A [76]	*A. niger*	$C_{24}H_{22}N_2O_5$		418.1529
10,23-Dihydro-24,25-dehydro-21-oxo-aflavinine [120]	*A. tubingensis* ATCC 76608	$C_{28}H_{37}NO_2$	224(18,200), 267(4,200), 273(1,400), 283(2,100), 291(1,750)	419.2824
14-epi-14-hydroxy-10,23-dihydro-24,25-dehydroaflavinine [122]	*A. tubingensis* ATCC 76608	$C_{28}H_{39}NO_2$		421.2975
14-epi-14-hydroxy-10,23-dihydro-24,25-dehydro-aflavinine [120]	*A. tubingensis* ATCC 76608	$C_{28}H_{39}NO_2$	226(16,400), 284(2,290), 292(2,100)	421.2980
Demethylkotanin [75]	*A. niger*	$C_{23}H_{20}O_8$		424.1153
Aspernigerin [73]	*A. niger*	$C_{26}H_{32}N_4O_2$		432.2525
Kotanin [56]	*A. niger*	$C_{24}H_{22}O_8$	203(92%), 208(100), 237(35), 259(15%), 296(36%), 306(41%), 317(35%)	438.1309
Aspernigrin B [57, 96]	*A. niger*	$C_{27}H_{24}N_2O_5$		456.1679
Yanuthone D [77]	*A. niger, A. tubingensis*	$C_{28}H_{38}O_8$	226(7,940), 254(3,715), 298(1,150)	502.2567
Malformin A_2 [63, 67]	*A. niger, A. brasiliensis, A. tubingensis*	$C_{22}H_{37}O_5N_5S_2$	End abs.	515.2239
Malformin B_5 [63, 67]	*A. niger, A. brasiliensis, A. tubingensis*	$C_{22}H_{37}O_5N_5S_2$	End abs.	515.2239
Malformin B_2 [63, 67]	*A. niger, A brasiliensis, A. tubingensis*	$C_{22}H_{37}O_5N_5S_2$	End abs.	515.2239
Yanuthone E [77]	*A niger, A. tubingensis*	$C_{29}H_{42}O_8$	234(9,120), 280(1,000), 340(450)	518.2881
Malformin A_1 [63]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
Malformin B_{1a} [63, 67]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
Malformin B_{1b} [63, 67]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
Malformin B_5 [63, 67]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
Malformin B_3 [63, 67]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
Malformin C [123]	*A. niger, A brasiliensis, A. tubingensis*	$C_{23}H_{39}O_5N_5S_2$	End abs.	529.2396
8′-*O*-Demethylnigerone [49]	*A. niger*	$C_{31}H_{24}O_{10}$	284 (56,200), 365 (12,020), 400 (9,775)	556.1369
8′-*O*-Demethylisonigerone [49]	*A. niger*	$C_{31}H_{24}O_{10}$	281 (33,885), 355 (26,915), 389 (7,760)	556.1369
Asperpyrone A [124]	*Nigri*	$C_{31}H_{24}O_{10}$,		556.1369
Dianhydroaurasperone C [125]	*Nigri*	$C_{31}H_{24}O_{10}$	225(22,908), 255(26,915), 280(85,113), 325(3,715), 405(5,754)	556.1369
6′-*O*-Demethylnigerone [106]	*Nigri*	$C_{31}H_{24}O_{10}$	226(39,810), 278(63,095), 408(10,000)	556.1369
Aurasperone D [44, 48, 102, 108, 109]	*Nigri*	$C_{31}H_{24}O_{10}$	235–240(50,119), 280(51,286), 320–325(15,136), 380(7,080)	556.1369
Carbonarin C [114]	*A carbonarius*	$C_{33}H_{27}NO_8$		565.1737
Carbonarin D [114]	*A carbonarius*	$C_{33}H_{27}NO_8$		565.1737
Carbonarin H [114]	*A. carbonarius*	$C_{33}H_{26}O_9$		566.1577
Isonigerone [49]	*Nigri*	$C_{32}H_{26}O_{10}$	UV: 226 (65%), 275 (100%), 327 (17%), 407 (19%)	570.1526
Asperpyrone B [124]	*Nigri*	$C_{32}H_{26}O_{10}$		570.1526
Asperpyrone C [124]	*Nigri*	$C_{32}H_{26}O_{10}$		570.1526
Aurasperone A [47, 102, 108, 109]	*Nigri*	$C_{32}H_{26}O_{10}$	225(51,300), 258(53,700), 280(100,000), 325(8,710), 406(12,880)	570.1526
Fonsecinone A [126]	*Nigri*	$C_{32}H_{26}O_{10}$	228(45,700), 256(44,670), 278(67,600), 325(11,480), 398(7,080)	570.1526
Fonsecinone D [126]	*Nigri*	$C_{32}H_{26}O_{10}$	227(26,900), 279(48,975), 316(8,128), 328(6,920), 403(6,310)	570.1526
Isoaurasperone A [44, 112]	*Nigri*	$C_{32}H_{26}O_{10}$	255(33,884), 275(38,905), 385–390(3,467)	570.1626

Table 2 (continued)

Component	Species	Elementary composition	UV/VIS	Monoisotopic mass (Da)
Isonigerone [49, 106]	*Nigri*	$C_{32}H_{26}O_{10}$	228(50,119), 248(56,234), 279(66,069), 390(9,550)	570.1626
Nigerone [106]	*Nigri*	$C_{32}H_{26}O_{10}$	226(51,286), 278(83,186), 407(14,125)	570.1626
Nigerasperone C [111]	*Nigri*	$C_{31}H_{26}O_{11}$	232 (50%), 282 (100%), 329 (15%), 403 (15%)	574.1470
Aurasperone F [48]	*Nigri*	$C_{31}H_{26}O_{11}$	213, 281, 320, 334	574.1475
Carbonarin A [114]	*A. carbonarius*	$C_{33}H_{26}O_{10}$		582.1526
Carbonarin B [114]	*A. carbonarius*	$C_{33}H_{26}O_{10}$		582.1526
Diketopiperazine dimer [68]	*A. tubingensis*	$C_{33}H_{38}N_6O_4$	240, 300	582.2949
Aurasperone G [48]	*Nigri*	$C_{31}H_{24}O_{12}$	UV: 213, 281, 315, 332, 403	588.1268
Aurasperone E [44]	*Nigri*	$C_{32}H_{28}O_{11}$	230(72,444), 282(147,910), 322(22,387), 330(20,418), 400(19,500)	588.1631
Fonsecinone B [126]	*Nigri*	$C_{32}H_{28}O_{11}$	229(38,900), 255(41,700), 280(70,800), 320(14,125), 328(14,125), 403(9,120)	588.1631
Fonsecinone C [126]	*Nigri*	$C_{32}H_{28}O_{11}$	234(51,285), 254(38,000), 279(60,255), 315(19,500), 327(14,800), 398(7,080)	588.1631
Aurasperone C [44, 47, 102, 108, 109]	*Nigri*	$C_{31}H_{28}O_{12}$	236, 283.5, 323, 336, 412	592.1581
Carbonarin G [114]	*A. carbonarius*	$C_{34}H_{28}O_{10}$		596.1682
10,10′-Bifonsecin B [49]	*Nigri*	$C_{32}H_{30}O_{12}$	231 (84%), 276 (100%), 330 (26%), 411 (24%)	606.1732
Nigerasperone B [111]	*Nigri*	$C_{32}H_{30}O_{12}$	236 (98%), 281 (100%), 316sh (32%), 380 (12%)	606.1732
Aurasperone B [44, 47, 48, 108, 109]	*Nigri*	$C_{32}H_{30}O_{12}$	235(51,000), 282(87,700), 321(20,100), 334(20,000), 410(13,600)	606.1732
Carbonarin F [114]	*A. carbonarius*	$C_{34}H_{28}O_{11}$		612.1632
Asperazine [64]	*A. tubingensis*	$C_{40}H_{36}N_6O_4$	225, 275, 300	664.2798
Fumonisin B_4 [11]	*A. niger*	$C_{34}H_{59}NO_{13}$	Not UV active	689.3986
Fumonisin B_2 [10]	*A. niger*	$C_{34}H_{59}NO_{14}$	Not UV active	705.3930
Biotransformation products (not true metabolites)				
Differenol A (genistein, prunetol, sophoricol) [127]		$C_{15}H_{10}O_5$		270.0528
3′,4′,5,7-Tetrahydroxy-8-methoxy isoflavone [128]		$C_{16}H_{12}O_7$		316.0578
Pisolithin B [88]		$C_8H_8O_4$		168.0417
8-Hydroxygenistein [128]		$C_{15}H_{10}O_6$		286.0472
Flaviolin [129]		$C_{10}H_6O_5$		206.0215
Orobole [127]		$C_{15}H_{10}O_6$		286.0472
Iso-T-2 toxin (Antibase2008)		$C_{24}H_{34}O_9$		466.2197

Extinctions coefficient

The identities of species marked with a *question mark* have not been confirmed.

[a] Series *Nigri*: *A. niger, A. tubingensis, A. acidus, A. brasiliensis, A. carbonarius, A. sclerotiivarbonarius, A. sclerotioniger, A. ibericus, A. vadensis, A. costaricaensis, A. piperis*

[b] This isolate produces an abundance of sclerotia, but has not been unequivocally placed in *A. tubingensis* yet.

End abs. End absorption (UV max. < 200nm)

higher mass resolution and 10–100 times better sensitivity than the LC-TOFMS instrument used here.

ESI of OTA mainly yields the protonated molecular ion $[M+H]^+$ in positive mode and $[M-H]^-$ in negative mode, with approximately 10 times higher signal in positive mode. With high in-source fragmentation settings, OTA yields several diagnostic ions for further confirmation. An unidentified fragment ion at *m/z* 358 can be observed along with the sodium adduct $[M+Na]^+$ and the ^{37}Cl $[M+H]^+$ at *m/z* 406 [19]. Care should be taken when using nominal-mass LC-MS, since a very common contaminant from plasticware (presumable a phthalate) with a molecular mass of 386 Da is eluted very close to OTA and makes at predominant $[M+NH_4]^+$ ion at *m/z* 404. OTA can be differentiated from the contaminant on the basis of the chlorine isotope pattern.

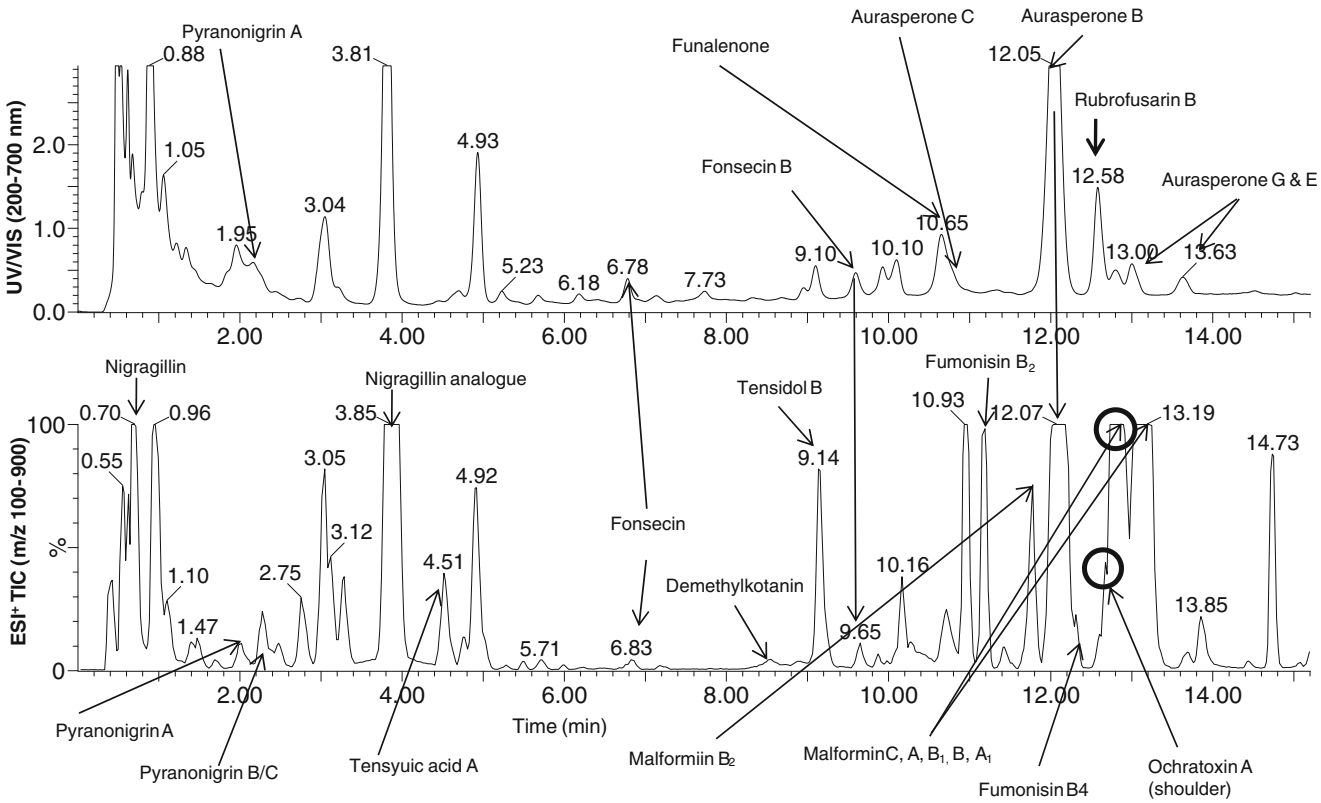

Fig. 2 *Aspergillus niger* extract from 8-day culture on yeast extract sucrose agar analyzed by liquid chroamtography–diode-array detection–time-of flight mass spectrometry. The *upper trace* is the UV/vis (200–700 nm) chromatogram and the lower trace is the total ion chromatogram (*TIC*) [positive electrospray ionization (ESI^+), *m/z* 100–900]. Separation was done using a Luna C_{18} II column with 15% CH_3CN to 100% CH_3CN in 20 min

Fumonisins

The fumonisins are a group of polyketide-derived mycotoxins, first discovered in 1988 from *F. verticillioides* [36]. This group of compounds is of great importance as they are suspected to be carcinogenic to humans, and are as a consequence regulated mainly in maize-based products. The fumonisins can be divided into four series A, B, C, and P [37], with the B series, mainly FB_1, FB_2, and FB_3 (Fig. 1), as the most abundant naturally occurring fumonisins [37, 38]. The surprising discovery of putative homologues to the *F. verticillioides* fumonisin gene cluster in two different *A. niger* genomes [13, 26] led to the subsequent documentation of actual FB_2 production in *A. niger* [10, 11]. Besides FB_2, FB_4 is also produced in lower amounts, about 10–25% of the amount of FB_2. From a current screening project on grapes and raisins it appears that fumonisins are produced by approximately 75% of all *A. niger* strains, whereas for coffee the figure was 76%, and they are thus much more common within *A. niger* than OTA production (6–10% [7, 8]).

Analysis of fumonisins produced by *Fusarium* spp. has previously been extensively reviewed [29, 37, 38]. In this review, FB_2 and FB_4 will therefore only be dealt with briefly in respect to the emerging data from analysis of *A. niger* infected commodities for which new analytical methods needs to be developed. Owing to the lack of suitable chromophores or flourophores, fumonisins cannot be detected directly using UV detection or FLD. This is probably why they were not detected in culture extracts of *A. niger* related species until recently. The fumonisins ionize excellently in positive ESI (ESI^+) and also quite well in negative ESI (ESI^-), yet about approximately tenfold lower than in ESI^+ using our instruments. Bartok et al. [37] have listed comprehensive LC-MS/MS data on the fumonisins.

With the increasing sensitivity of LC-MS/MS, especially with triple quadrupole mass spectrometers, multimethods with no purification are emerging for low parts per billion levels of well-ionizing mycotoxins. An example is the screening method of Sulyok et al. [39, 40] which was used to detect fumonisins down to 3–17 μg/kg and OTA down to approximately 1 μg/kg. The method was used to detect very high levels of FB_2 (33 mg/kg) in dark bread, where the absence of FB_1 as well as a black infecting fungus on the bread together indicate that the contaminant was an *A. niger* rather than a *Fusarium* species. FB_2 and FB_4 probably produced by *A. niger* were also reported in [41] to have been detected by LC-TOFMS.

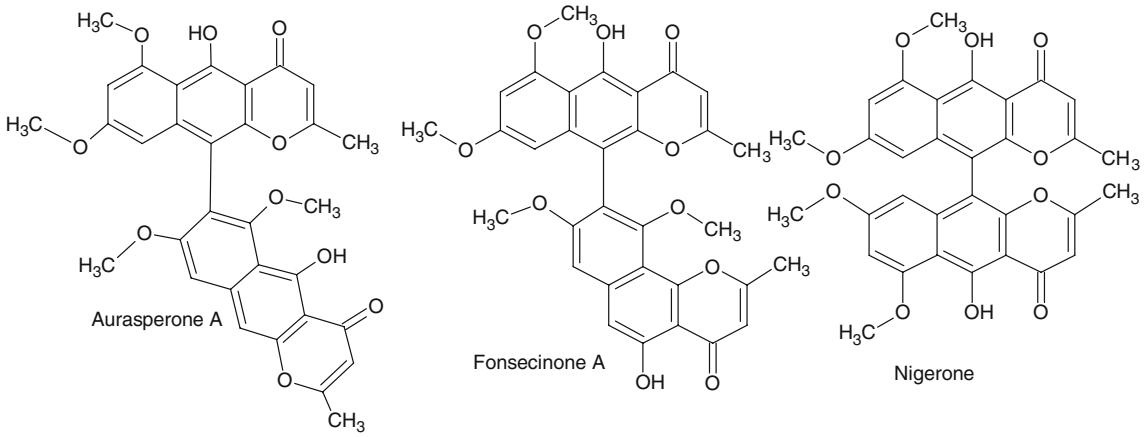

Fig. 3 Structures representing the three major groups of bis(naptho-γ-pyrone)s in the *A. niger* group

From CH_3CN extracts of strawberries, we were able to detect fumonisins with LC-MS/MS down to 10 μg/kg and OTA down to 1 μg/kg, and on berries artificially infected with *A. niger* we found up to 25 mg/kg FB_2 and 2 mg/kg FB_4, as well as up to 1 μg/g OTA, whereas malformins were barely detected (K.F. Nielsen, unpublished results).

For analysis of green coffee, the EN 13585:2001 protocol (70% CH_3OH, strong anion exchange, SAX) for extraction and purification of fumonisins in maize could be directly adapted [11]. Furthermore, OTA which cooccurred in the extract was simultaneously retained and detected by LC-MS/MS [21]. Immunoaffinity purification of fumonisins was not a successful strategy for extracts from green and roasted coffee and resulted in viscous dark samples. A commercial ELISA kit (developed for maize) was also tested, but in green coffee it gave totally random results with many false positives and absolutely no correlation to LC-MS/MS results [11].

Since some *A. niger* strains produce very high amounts of citric acid and other small organic acids (up to 50 g/L) under some conditions, it has not always been possible to use SAX purification. Recoveries from SAX have been seen as low as 0% for OTA and fumonisins owing to competition with small organic acids. In consequence, we are currently testing strong cation exchange. Surprisingly, we have not found publications on cation-exchange purification of fumonisins, which is probably a reflection of the first fumonisin methods, where derivatization with *O*-phthalaldehyde and subsequent LC-FLD was the preferred analytical strategy [29, 37, 38]. And since both *O*-phthalaldehyde and cation exchange target amines, these two methods are not orthogonal and thus not selective as a pair. However, with LC-MS as the detection principle, cation exchange should statistically be preferred since there are far fewer basic than acidic compounds in microorganisms.

Naptho-γ-pyrones

Quantitatively, the NGPs represent the most abundant family of secondary metabolites in the *A. niger* group under all conditions observed (including chemostate cultures).

The biological effects of several NGPs have been investigated in various systems [14], and they have, for instance, been shown to be antibacterial, antifungal [42], antitumoral [43], and cytotoxic [42, 43]. Ghosal et al. [44] reported acute toxicity (interperitoneal) at the 10–50 mg/kg level; however, to the best of our knowledge no data on the bioavailability of these compounds exist. Thus, these compounds cannot currently be considered mycotoxins *sensu stricto*, since this requires toxicity via a natural route of exposure.

The NGP group of compounds comprises a series of aurasperones, fonsecinones, and nigerones, as well as monomers such as flavasperone and rubrofusarin B (Figs. 3 and 4). Reanalysis of in-house LC-DAD and LC-MS data showed

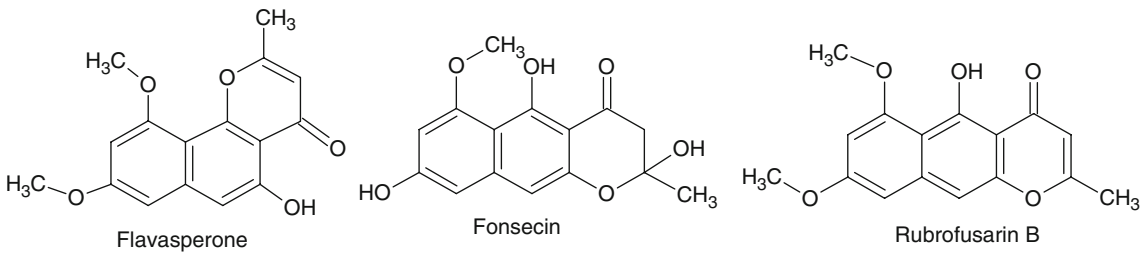

Fig. 4 Structures representing the three major groups of monomeric naptho-γ-pyrones in the *A. niger* group

that all species within the "*A. niger* clade" are able to produce NGPs (*A. acidus, A. brasiliensis, A. carbonarius*, *A. costaricaensis*, *A. ibericus*, *A. niger*, *A. piperis*, *A. sclerotiicarbonarius*, *A. sclerotioniger*, *A. tubingensis,* and *A. vadensis* [5]), whereas species not producing NGPs include the "*A. ellipticus/A. heteromorphus* clade" and the "*A. aculeatus* clade" (comprising *A. homomorphus, A. aculeatinus, A. aculeatus, A. japonicus*, *and A. uvarum*). However, in losing the ability to produce black conidia, *A. lacticoffeatus*, a light-brown-spored naturally occurring mutant of *A. niger*, has also lost the ability to produce NGPs, which is also the case for white-spored industrial strains we have analyzed. So there appears to be a link between black melanin production and NGP production in the *A. niger* group. Moreover, NGPs have been shown to be present in aerosolized spores [45].

Of 140 *A. niger* isolates we have analyzed by LC-DAD and LC-DAD-TOFMS, 134 (96%) were found to produce at least one NGP (usually aurasperone B in the highest amount). Comparable levels were seen for *A. tubigensis* (169 of 177, 95%), *A. ibericus* (three of three, 100%), and *A. acidus* (44 of 47, 94%) [46]. These data are thus conflicting with data reported by Bouras et al. [47], who only found NGPs in a low proportion of the strains investigated. This can be ascribed to differences in strain age, medium, and incubation conditions, since it has been our experience that the NGP levels are highest in fresh isolates and may decrease somewhat over time.

With LC-ESI-TOFMS, simple ionization patterns have been observed. In ESI^+, $[M+H]^+$ was the most abundant ion, and $[M+Na]^+$ and $[M+Na+CH_3CN]^+$ were the main adduct ions. In ESI^-, mainly $[M-H]^-$ and low-abundance $[M-2H+Na]^-$ ions was observed, the latter ion matching an acidic functionality (here a phenol group). Identical patterns were observed by Bouras et al. [47, 48], who also reported numerous specific fragment ions (aurasperones F and G) from MS/MS experiments (ion trap). These can also serve as potential MRM transitions for trace analysis.

The NGPs contain a fully conjugated system, giving rise to very characteristic UV/VIS spectra (Table 2, Fig. 5). However as seen in Table 2, and in the figures in Zhang et al. [49], some spectra are similar, and for the ones with the same elementary composition (e.g., ten different compounds with the formula $C_{32}H_{26}O_{10}$) it may even be difficult to separate them by their MS/MS spectra as they are mostly positional isomers which will probably fragment in a similar way. Thus, the retention time is the only way to differentiate NGPs if no reference standards are available or if no NMR validation can be done. An alternative is to compare the whole profiles with the one in Fig. 2 and the ones in Zhang et al. [49], since the order of elution of the NGPs should be the same if low-pH reversed-phase LC is used.

Since the NGPs constitute a large proportion of the total peak area and they absorb strongly in the 200–450-nm region, they often obscure the detection of compounds such as the malformins and ochratoxins. During preparative isolation of fumonisins, it has been observed that NGPs are not retained on SAX columns, but are retained on mixed-mode reversed-phase SAX columns (e.g., Oasis MAX), as secondary interactions with a polymeric backbone will cause strong retention of these weakly acidic phenols [50, 51]. These columns can then serve as a prefractionation step to selectively remove the NGPs.

Study of the occurrence of NGPs and other related compounds in food and feed samples is limited to the study of Ghosal et al. [44], who detected flavasperone, rubrofusarin, isoaurasperone, and aurasperones E, A, and D in artificially infected mangos at a total level of 60 mg/kg. NGPs were detected using thin-layer chromatography (280-nm absorption) after organic extraction and aurasperones A and D were found to be the predominant ones (approximately 33% each). Aurasperones (erroneously named tetracyclic compounds [52]) and orlandin were also found from *A. niger* infected building materials [53]. Since other metabolites originating

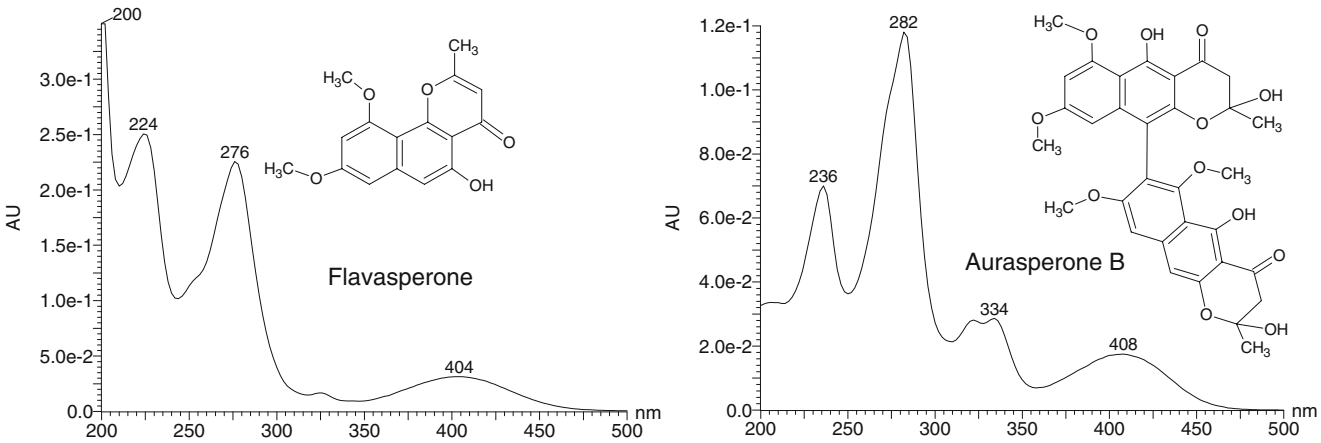

Fig. 5 UV spectra (pH 3.2) of monomeric (flavasperone) and dimeric (aurasperone B) naptho-γ-pyrones

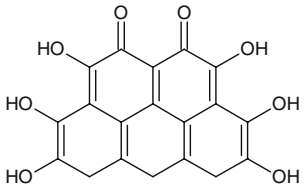

Fig. 6 Structure of the green pigment aspergillin

from the *A. niger* group can be found in food (OTA and FB_2), NGPs are most likely to be naturally occurring in various food matrices, and hereby it would be recommendable to develop analytical protocols for detection of NGPs in such food types as well as investigating their bioavailability and subsequent toxicity.

Occasionally, we have been asked to analyze yellow samples from submerged fermentations with different, mainly industrial *A. niger* strains. With LC-DAD-TOFMS we have each time detected aurasperone B as the only secondary metabolite in the sample. In most cases, ESI^- has been necessary for MS verification since foam suppressors [silicon and/or poly(ethylene glycol) oils] also suppress ESI^+ signals.

In addition, a green pigment has been observed in *A. niger* fermentations. This is aspergillin (Fig. 6), a highly oxygenated polyketide with a hexahydroxyl pentacyclic quinoid structure [54]. Together with another kind of melanin made from monomers, the black pigment is made up from a complicated mixture of aspergillin and proteins [54].

Bicoumarins

The bicoumarins (Fig. 7) represent another prominent family of compounds from *A. niger*, consisting of a group of heterocyclic dimers derived from cinnamic acid lactone, further categorized on the basis of the type of connection between the coumarin moieties. Like the NGPs, the bicoumarins contain a fully conjugated system and thus have very characteristic UV absorption profiles (Fig. 8) that make them easy to tentatively identify in an extract as a group. However, many variations with the same molecular formula make it difficult to make a positive identification on the basis of UV or MS data alone. Bicoumarins seems to be present in most *A. niger* strains, although they sometimes coelute with the aurasperones, which can obscure their detection. Orlandin, kotanin, and desmethyl-orlandin (based on LC-DAD, and LC-DAD-TOFMS) are consistently produced by *A. niger and A. tubingensis.* Another polyketide is funalenone [55], which is consistently (based on LC-DAD-TOFMS) produced by *A. niger* but also by *A. tubingensis* and *A. brasiliensis.*

The bicoumarins have been found to have some inhibitory effects on plant growth [56] and a moderate cytotoxic effect in human cell lines in vitro [57], but at present they are considered nontoxic [56, 59]. Yet, potential bioactivities of compounds of this type have not been investigated much and as a consequence no methods and data exist for their analysis.

Malformins

The malformins (Fig. 9) got their name owing to their ability to cause malformation in plants [60], and have also been reported to be antibacterial [61] and to inhibit interleukin-1β binding to various human cells [62]. The malformins, which are cyclic pentapeptides with a sulfur bridge, have often been called mycotoxins since they have been shown to be toxic after peritoneal injection. However, since they were not toxic by oral administration [63] they cannot currently be considered mycotoxins *sensu stricto.*

In the *A. niger* group, only *A. brasiliensis*, *A. niger*, and *A. tubingensis* produce malformins [5, 64, 65], with *A. brasiliensis* probably being the best producer (confirmed by both LC-DAD-TOFMS and LC-MS/MS). Since they are produced by several abundant food contaminants, a

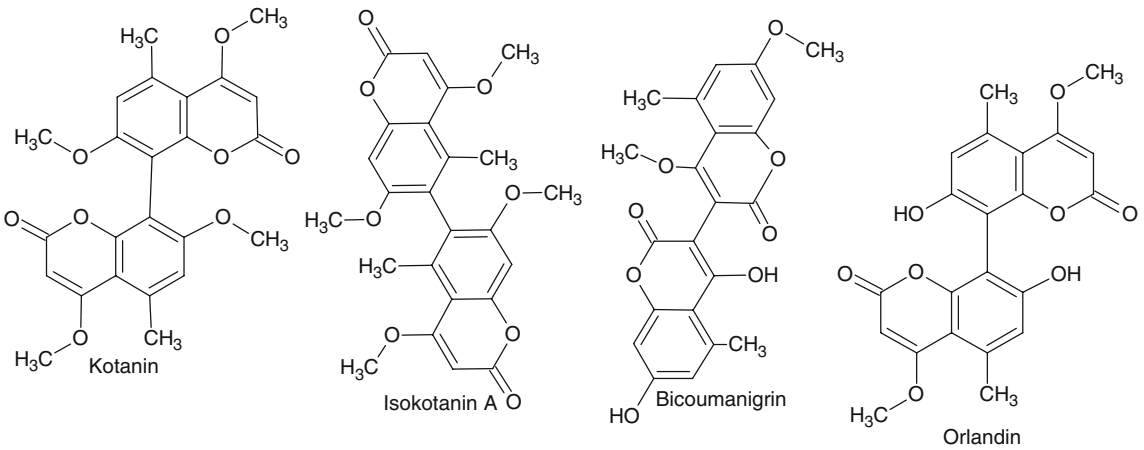

Fig. 7 Structure of selected bicoumarins

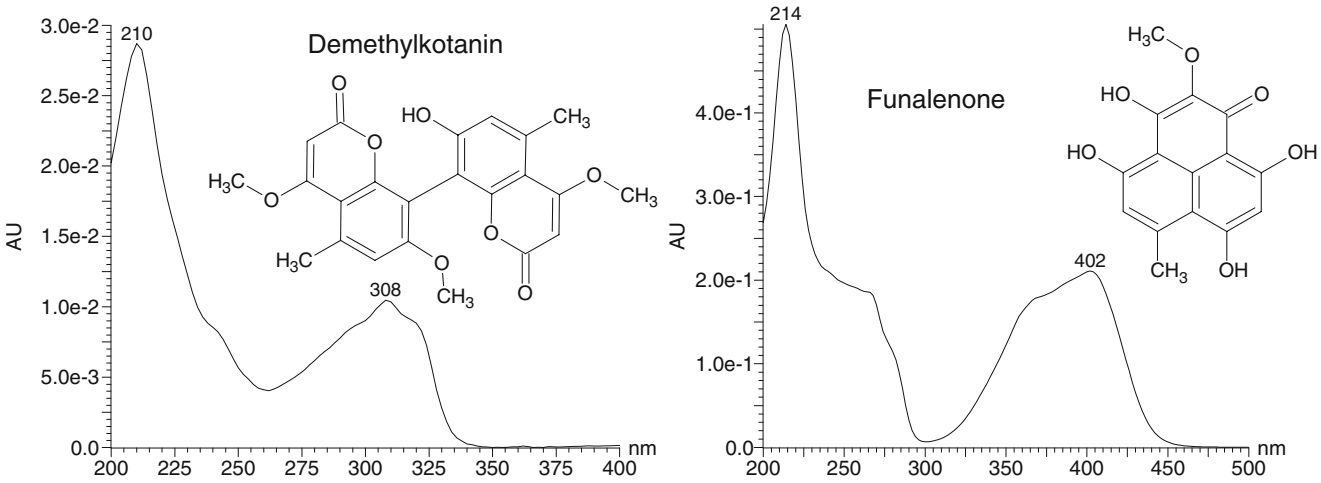

Fig. 8 Structure and UV spectrum of selected bicoumarins

toxicological evaluation, including bioavailability testing, is highly needed.

Even though malformins can be detected using LC-UV detection [19], in our experience they cannot be detected in *Aspergillus* extracts using this method owing to many interfering peaks. That is why LC-MS is the obvious choice for their detection [19, 66]. The sulfur bridge should also enable high-sensitivity electrochemical detection.

Chromatographic conditions used for analysis of the malformins are all based on acidic reversed chromatography [19, 66, 67], as normal phase and paper LC were not efficient [67]. Kim et al. [67] used a purification scheme based on acidic isocratic water–methanol (35:65 v/v) LC on an octadecyl silica column.

In ESI^+, the malformins have a high tendency to form $[M+NH_4]^+$, $[M+Na]^+$, and $[M+K]^+$ adducts [19], often with the abundance of $[M+H]^+$ 20% lower than that of the $[M+NH_4]^+$ ion. If high in-source fragmentation settings are used, sodium and potassium adducts will dominate. In ESI^+, *m/z* 417 is often observed from in-source fragmentation, and it is also a specific fragment for MS/MS analysis (MRM) along with *m/z* 372 and 304. In ESI^-, the malformins do not ionize as well as in ESI^+ (tested on both MS instruments used) and are mainly detected as the $[M+HCOO]^-$ and $[M+Cl]^-$ adducts, with the abundance of $[M-H]^-$ 20% lower than that of the other two adducts.

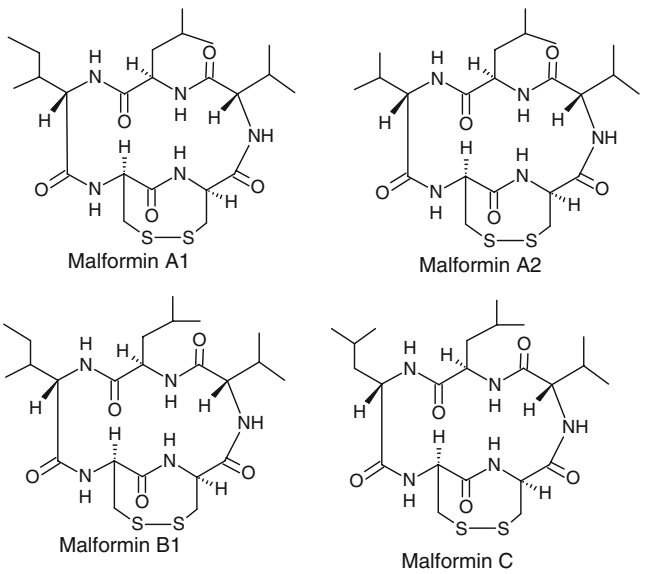

Fig. 9 Structures of selected malformins

Asperazines

Asperazine (Fig. 10) is a complex diketopiperazine dimer, first isolated from a marine-derived *A. tubingensis* (reported as *A. niger)* by Varuglu et al. [64]. An analogue was later described by Ovenden et al. [68], also from an *A. tubingensis* strain. Asperazine was previously found to be cytotoxic in vitro against leukemia [64], but a later investigation reported it to be noncytotoxic [69].

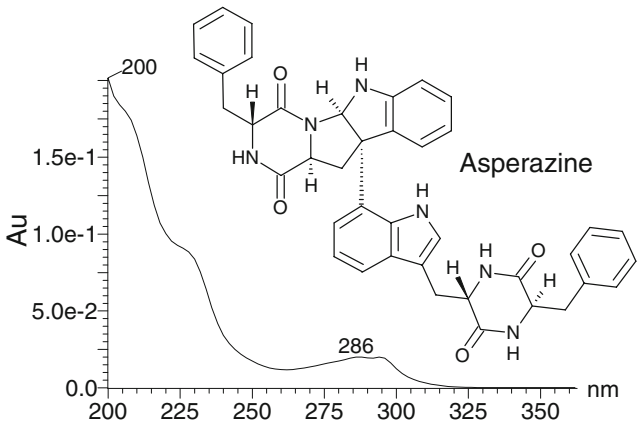

Fig. 10 UV spectrum and structure of asperazine

Reanalysis of extract data (approximately 66% by LC-TOFMS and all by LC-FLD) from 140 *A. niger*, 177 *A. tubingensis*, one *A. vadensis* [5], and 47 *A. acidus* (*A. foetidus*) [46, 70] strains for the production of asperazine showed that none of the *A. niger* strains produced asperazine, whereas a consistent production was observed in *A. acidus* and *A. tubingensis*. Owing to this limited distribution within the group, asperazine seems to be a valuable chemical marker that can be used to distinguish less toxic species such as *A. acidus* and *A. tubingensis* from otherwise similar but more toxic species such as *A. niger*.

As most alkaloids, asperazines ionize very well in ESI^+, with $[M+H]^+$ as the only ion detected. Furthermore, asperazines show strong fluorescence at 230–450 nm (acidic conditions), and can usually also be detected from their distinct UV spectra using LC-DAD (UV/vis) (Fig. 10). Only acidic reversed-phase separations have been reported for their separation [5, 64, 70].

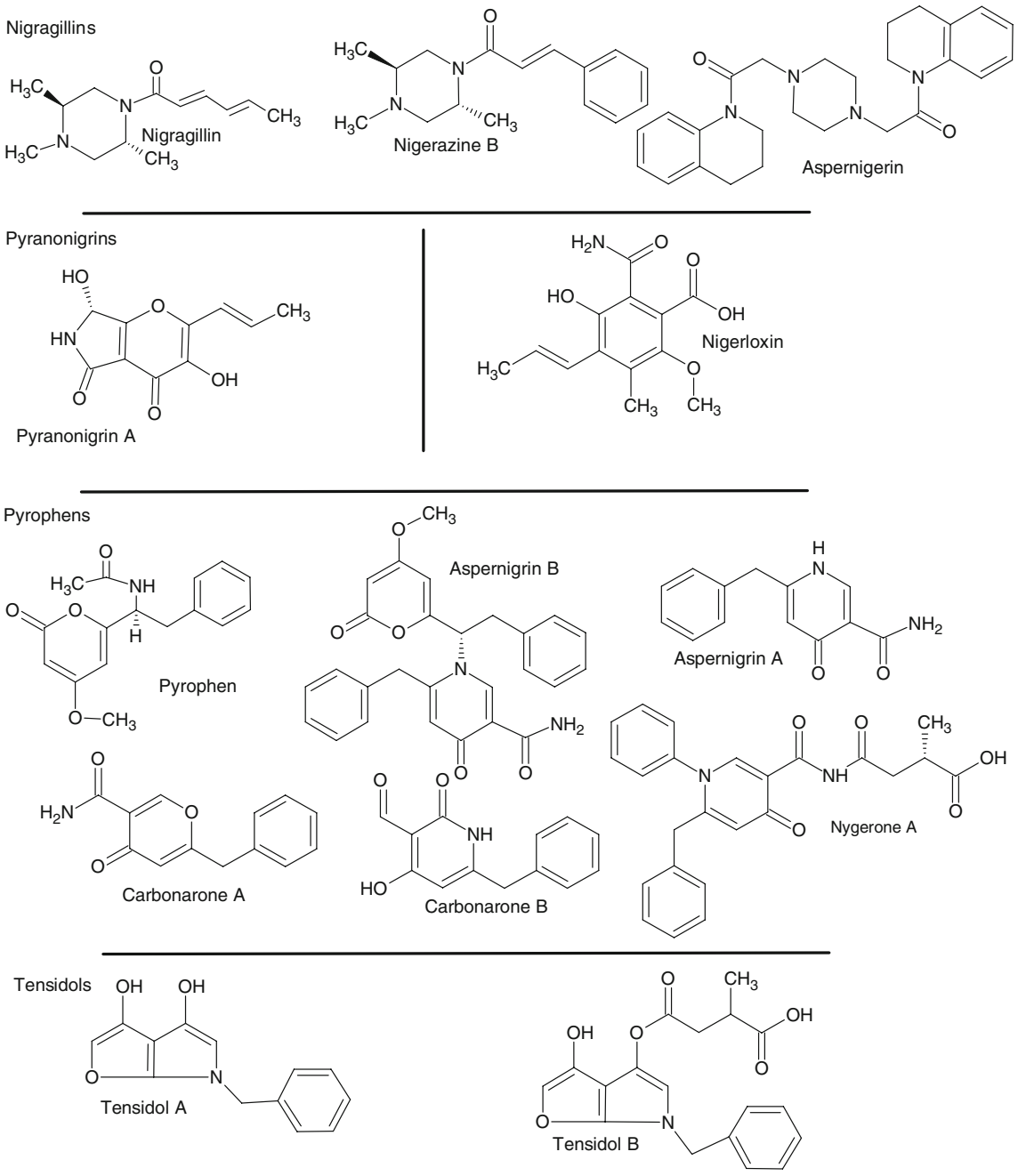

Fig. 11 Structures of the five classes of alkaloids

Other alkaloids

Species in the *A. niger* group produce a number of other nitrogen-containing compounds besides the fumonisins, ochratoxins, malformins, and asperazines. These alkaloids can be grouped into nigragillins, pyranonigrins, nigerloxin, pyrophens, and tensidols (Fig. 11).

Very few data are available for these compounds, with respect to both biological activities and which species they are found in. To our knowledge no studies exist on their presence in food and feeds, which is no surprise since they are, as is also the case for the NGPs, bicoumarins, and malformins, not commercially available.

Owing to their inherent properties as alkaloids, all of the compounds in this class have been found to ionize very well in ESI^+ and form strong $[M+H]^+$ ions, and except for the tensidols, they have very poor or no signal in ESI^-. In addition, many of them have characteristic chromophores suitable for LC-DAD.

The nigragillin-like group of alkaloids in the black aspergilli comprise nigragillin, nigerazines A and B [14, 71], aspernigrin B [57, 72], and aspernigerin [73]. Previous reports on nigragillin production within the *A. niger* group are restricted to *A. niger* (reported as *A. phoenicis*) [74]. Nigragillin was found on *A. niger* infected building materials, where the overall profile appeared similar to that of most rich-solid agar-based substrates [53].

We have found that nigragillin is easy to detect using LC-DAD-TOFMS, forming $[M+H+CH_3CN]^+$ besides $[M+H]^+$. Notably, to obtain retention in reversed-phase LC, the start gradient should not exceed more than 10% CH_3CN.

Results based on LC-DAD and LC-TOFMS show that all the species (but not all strains) in the "*A. niger* clade" produce pyranonigrins, mainly as pyranonigrin A [5]. The structure of pyranonigrin was first reported by Hiort et al. [57], who later revised it [58] and recently Schlingmann et al. [75] revised it again.

Pyranonigrin ionizes strongly in ESI^+ as $[M+H]^+$, with some $[M+Na]^+$ and $[M+Na+CH_3CN]^+$ adducts also being formed. Owing to the relatively high polarity of these compounds, it is necessary to start a chromatographic gradient in the reverse phase at maximum 10% CH_3CN to obtain proper baseline separation from other early eluted compounds.

According to our data, pyrophens and tensidols are restricted to production in *A. tubingensis* and *A. niger*. On the basis of UV data, both produce several pyrophen analogues, all with the same ion pattern of $[M+H]^+$, some $[M+Na]^+$, and $[M+NH_4]^+$ adducts. The tensidols have consistently been detected in *A. niger* and *A. tubingensis* [15], where tensidol B is produced in much higher quantities than tensidol A. They ionize strongly in ESI^+ as $[M+H]^+$, with some $[M+NH_4]^+$ and $[M+Na+CH_3CN]^+$ ions. Tensidol A loses the 2-methylbutanedioic acid moiety to form fragment *m/z* 230. In ESI^- $[M-H]^-$ was observed along with some $[M-H+H_2O]^-$ which must be formed via hydrolysis of one of the double bonds.

A novel analogue of pyrophen, nygerone A, was recently described by Henrikson [76] as a cryptic gene product which was induced by addition of histone inhibitors. Potentially, more new metabolites from *A. niger* or other black aspergilli could be unveiled by accessing cryptic gene clusters.

Remaining compounds

Very few terpenes have been isolated from the *A. niger* group, and they all contain a polyunsaturated chain as seen in Fig. 12. No data exist on the natural occurrence of terpenoids and investigation of biological activity has mainly been restricted to antimicrobial activities [77].

Reports of gibberellic acid [78] seem to be unlikely as the identity was only confirmed by a simple unspecific spectrometric assay. Another example is nigerloxin [79], where we have not found the compound in any extracts from the whole group when searching data files of approximately 150 extracts analyzed by LC-TOFMS for the $[M+H]^+$, $[M+Na]^+$, and $[M+H-NH_3]^+$ ions. The latter ion was included since screening of our reference standard

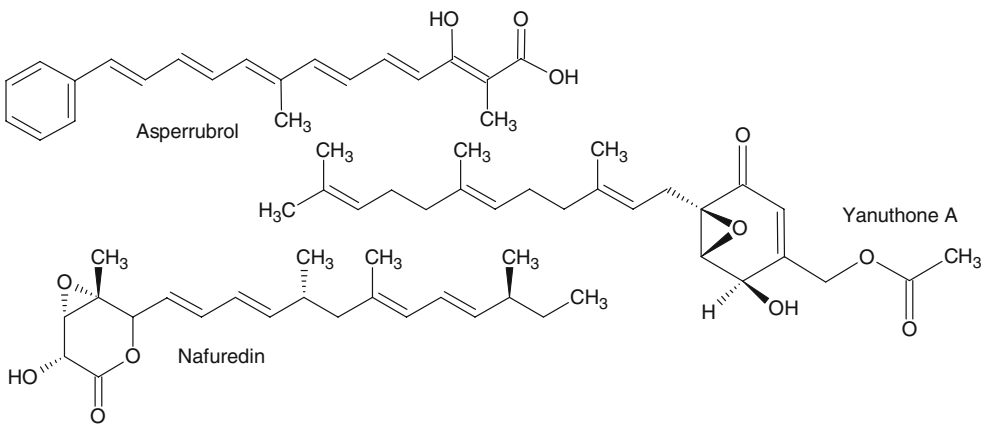

Fig. 12 Structure of selected terpenes

library has shown that NH_3 loss (using in-source fragmentation) is only observed from primary amides.

Biotransformation

This review is not intended to cover this area; however, it needs to be mentioned as some papers do not clearly state that the compounds detected from, for example, *A. niger* are actually biotransformed (small chemical changes to a complex molecule from other species) rather than fully biosynthesized by the fungus. Some of the well-known compounds derived from biotransformation are mentioned at the end of Table 2 and include compounds from animal feed or transformation of the feed material (genisteins, the flavonoids, isoflavone, pisolithin B, orobole), whereas iso-T-2 toxin is a transformation of the well-known *Fusarium* toxin T-2 toxin fed to the culture.

Analytical methods for mycotoxin screening

Summarizing the analytical observations, it appears that validated analytical methods only exist for OTA and the fumonisins. For the remaining compounds produced by the black aspergilli, the existing papers are mainly descriptions of the isolation procedure and structural elucidation, and no reference standards are commercially available. However, it should be possible to include them in an analytical scheme on a nonquantitative scale by using *A. niger* extracts for tuning MRM transitions when using triple quadrupole instruments, as, for example, is done for *F. avenaceum* metabolites in infected apples [80].

Profiles of culture extracts can also be compared with those of crude extracts of infected food and feed samples obtained using newer LC-TOFMS and Orbitrap instruments [41, 81]. These are getting increasingly more and more sensitive, and are a more interesting alternative to MRM analysis as they allow reprocessing of data files for more compounds [82]. With this approach, it is our recommendation to simultaneously monitor compounds within a series, for example, both FB_2 and FB_4, several of the malformins (A–C), or several NGPs, as compounds within a class are usually always coproduced. This will help pinpoint suspicious samples, where, for example, only one malformin is detected.

Given that these compounds will be produced in infected crops, monitoring of NGPs, tensidols, fumonisins, malformins, bicoumarins (kotanins), and asperazines should enable the detection of growth of black aspergilli to species level.

Even though LC combined with DAD and accurate mass determination is a strong tool for identifying mycotoxins and other fungal metabolites, a correct elementary composition, characteristic UV spectra, and the same elution profile [47, 83] are not sufficient for unambiguous identification of positional isomers of, for example, the NGPs or malformins. Detection in the original strain will provide a very strong tentative identification; however, for absolute identification NMR data are required. Improvements in the area of LC-NMR have made it an option even for the analytical chemist, as it is now possible to obtain data in the nanomole range [84]. Combining accurate mass determination with a few NMR recognizable features will make it possible to quickly identify positional isomers of known compounds [84].

In addition, it should be possible to quantify compounds in a fraction by NMR, since signals are proportional the number of moles in the tube. Subsequently one can calibrate against other (standardized) tubes containing accurate amounts of other compounds [85].

Conclusion

In conclusion, species within *Aspergillus* section *Nigri* are excellent producers of a large number of diverse secondary metabolites. Several new metabolites and maybe even new biosynthetic pathways are expected to be discovered in the near future now that the full genome of *A. niger* has been sequenced and soon also the full genomes of other black aspergilli will be sequenced.

Currently LC-DAD with accurate mass determination provides the easiest and most efficient strategy for tentative mapping of secondary metabolites in *A. niger* and its close relatives, especially if compared with already published chromatographic profiles, MS/MS data, and UV whole spectra. If absolute identification and positional isomer identification is needed, LC-NMR will be necessary.

For determination in food and feed, direct analysis of diluted crude extracts using LC-MS/MS analysis or LC-high-resolution mass-spectrometric detection is suggested. If sample pretreatment is needed, care should be taken when using anion exchange since *A. niger* can produce extremely high amounts of organic acids which can outsalt acidic target metabolites.

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References

1. Pitt JI, Hocking AD (1997) Fungi and food spoilage II. Blackie, London

2. Perrone G et al (2007) Stud Mycol 59:53–66
3. Frisvad JC et al (2007) Stud Mycol 59:31–37
4. Frisvad JC, Smedsgaard J, Larsen TO, Samson RA (2004) Stud Mycol 49:201–241
5. Samson RA, Noonim P, Meijer M, Houbraken J, Frisvad JC, Varga J (2007) Stud Mycol 59:129–145
6. Abarca ML, Accensi F, Cano J, Cabanes FJ (2004) Antonie Van Leeuwenhoek 86:33–49
7. Abarca ML, Bragulat MR, Castella G, Cabanes FJ (1994) Appl Environ Microbiol 60:2650–2652
8. Samson RA, Houbraken JAMP, Kuijpers AFA, Frank JM, Frisvad JC (2004) Stud Mycol 50:45–61
9. Esteban A, Abarca ML, Bragulat MR, Cabanes FJ (2006) Food Microbiol 23:634–640
10. Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U (2007) J Agric Food Chem 55:9727–9732
11. Noonim P, Mahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA (2009) Food Addit Contam 26:94–100
12. Schuster E, Dunn-Coleman N, Frisvad JC, Van Dijck PW (2002) Appl Microbiol Biotechnol 59:426–435
13. Pel HJ et al (2007) Nat Biotechnol 25:221–231
14. Blumenthal CZ (2004) Regul Toxicol Pharmacol 39:214–228
15. Varga J et al (2007) Int J Syst Evol Microbiol 57:1925–1932
16. Frisvad JC, Thrane U (1987) J Chromatogr 404:195–214
17. Smedsgaard J (1997) J Chromatogr A 760:264–270
18. Jennessen J, Nielsen KF, Houbraken J, Schnürer J, Lyhne EK, Frisvad JC, Samson RA (2005) J Agric Food Chem 53:1833–1840
19. Nielsen KF, Smedsgaard J (2003) J Chromatogr A 1002:111–136
20. Nielsen KF, Graefenhan T, Zafari D, Thrane U (2005) J Agric Food Chem 53:8190–8196
21. Noonim P, Mahakarnchanakul W, Nielsen KF, Frisvad JC, Samson RA (2008) Int J Food Microbiol 128:197–202
22. Johansen M (2007) MSc thesis. Technical University of Denmark, Lyngby
23. Medina A, Mateo R, Lopez-Ocana L, Valle-Algarra FM, Jimenez M (2005) Appl Environ Microbiol 71:4696–4702
24. Perrone G, Mule G, Susca A, Battilani P, Pietri A, Logrieco A (2006) Appl Environ Microbiol 72:680–685
25. Gomez C, Bragulat MR, Abarca ML, Minguez S, Cabanes FJ (2006) Food Microbiol 23:541–545
26. Bayman P, Baker JL (2006) Mycopathologia 162:215–223
27. Creppy EE (2002) Toxicol Lett 127:19–28
28. Stoev SD, Vitanov S, Anguelov G, Petkova-Bocharova T, Creppy EE (2001) Vet Res Commun 25:205–223
29. Krska R, Schubert-Ullrich P, Molinelli A, Sulyok M, MacDonald S, Crews C (2008) Food Addit Contam 25:152–163
30. Songsermsakul P, Razzazi-Fazeli E (2008) J Liq Chromatogr Relat Technol 31:1641–1686
31. Mateo R, Medina A, Mateo EM, Mateo F, Jimenez M (2007) Int J Food Microbiol 1:79–83
32. Dall'Asta C, Galaverna G, Dossena A, Marchelli R (2004) J Chromatogr A 1024:275–279
33. Saez JM, Medina A, Gimeno-Adelantado JV, Mateo R, Jimenez M (2004) J Chromatogr A 1029:125–133
34. Krska R, Molinelli A (2007) Anal Bioanal Chem 387:145–148
35. Zheng MZ, Richard JL, Binder J (2006) Mycopathologia 161:261–273
36. Gelderblom WCA, Jaskiewicz K, Marasas WFO, Thiel PG, Horak RM, Vleggaar R, Kriek NPJ (1988) Appl Environ Microbiol 54:1806–1811
37. Bartok T, Szecsi A, Szekeres A, Mesterhazy A, Bartok M (2006) Rapid Commun Mass Spectrom 20:2447–2462
38. Shepard GS (1998) J Chromatogr A 815:31–39
39. Sulyok M, Krska R, Schuhmacher R (2007) Anal Bioanal Chem 389:1505–1523
40. Sulyok M, Krska R, Schuhmacher R (2007) Food Addit Contam 24:1184–1195
41. Senyuva HZ, Gilbert J (2008) J Food Prot 71:1500–1504
42. Song YC, Li H, Ye YH, Shan CY, Yang YM, Tan RX (2004) FEMS Microbiol Lett 241:67–72
43. Koyama K, Ominato K, Natori S, Tashiro T, Tsuruo T (1988) J Pharmacobio-dyn 11:630–635
44. Ghosal S, Biswas K, Chakrabarti DK (1979) J Agric Food Chem 27:1347–1351
45. Palmgren MS, Lee LS (1986) Environ Health Perspect 66:105–108
46. Mogensen JM, Varga J, Thrane U, Frisvad JC (2009) Int J Food Microbiol 132:141–144
47. Bouras N, Mathieu F, Coppel Y, Strelkov SE, Lebrihi A (2007) J Agric Food Chem 55:8920–8927
48. Bouras N, Mathieu F, Coppel Y, Lebrihi A (2005) Nat Prod Res 19:653–659
49. Zhang YP, Ling S, Fang YC, Zhu TJ, Gu QQ, Zhu WM (2008) Chem Biodivers 5:93–100
50. Kanaujia PK, Pardasani D, Gupta AK, Kumar R, Srivastava RK, Dubey DK (2007) J Chromatogr A 1161:98–104
51. Shiomi K, Uchida R, Inokoshi J, Tanaka H, Iwai Y, Omura S (1996) Tetrahedron Lett 37:11265–1268
52. Shu Y-Z, Cutrone JQ, Klohr SE, Huang S (1995) J Antibiot 48:1060–1065
53. Nielsen KF, Gravesen S, Nielsen PA, Andersen B, Thrane U, Frisvad JC (1999) Mycopathologia 145:43–56
54. Ray AC, Eakin RE (1975) Appl Microbiol 30:909–915
55. Inokoshi J, Shiomi K, Masuma R, Tanaka H, Yamada H, Omura S (1999) J Antibiot. 52:1095–1100
56. Cutler HG, Crumley FG, Cox RH, Hernandez O, Cole PJ, Dorner JW (1979) J Agric Food Chem 27:592–595
57. Hiort J et al (2004) J Nat Prod 67:1543
58. Hiort J, Maksimenka K, Reichert M, Perovic-Ottstadt S, Lin WH, Wray V, Steube K, Schaumann K, Weber H, Proksch P, Ebel R, Muller WEG, Bringmann G (2005) J Nat Prod 68:1821 (Erratum to previous reference)
59. Buchi G, Klaubert DH, Shank RC, Weinreb SM, Wogan GN (1971) J Org Chem 36:1143
60. Takahashi N, Curtis RW (1961) Plant Physiol 36:30–36
61. Kobbe B, Cushman M, Wogan GN, Demain AL (1977) Appl Environ Microbiol 33:996–997
62. Herbert JM, Savi P, Lale A, Laplace MC, Baudry N, Pereillo JM, Emondsalt X (1994) Biochem Pharmacol 48: 1211–1217
63. Yoshizawa T, Tsuchiya Y, Morooka N, Sawada Y (1975) Agric Biol Chem 39:1325–1326
64. Varoglu M, Corbett TH, Valeriote FA, Crews P (1997) J Org Chem 62:7078–7079
65. Varoglu M, Crews P (2000) J Nat Prod 63:41–43
66. Senyuva HZ, Gilbert J, Ozturkoglu S (2008) Anal Chim Acta 617:97–106
67. Kim K-W, Sugawara F, Yoshida S, Murofushi N, Takahashi N, Curtis RW (1993) Biosci Biotechnol Biochem 57:787–791
68. Ovenden SP et al (2004) J Nat Prod 67:2093–2095
69. Govek SP, Overman LE (2007) Tetrahedron 63:8499–8513
70. de Vries EGE, Frisvad JC, van de Vondervoort PJI, Burgers K, Kuijpers AFF, Samson RA, Visser J (2005) Antonie Van Leeuwenhoek 87:195–203
71. Iwamoto T, Shima S, Hirota A, Isogai A, Sakai H (1983) Agric Biol Chem 47:739–743
72. Bringmann G, Maksimenka K, Gulder T, Schaumann K, Perovic-Ottstadt S, Mueller WEG, Hiort J, Ebel R, Proksch P (2004) Patent DE102004002884-A1
73. Shen L, Ye Y-H, Wang X-T, Zhu H-L, Xu C (2006) Chem Eur J 4395–4396

74. Cole RJ, Cox RH (1981) Handbook of toxic fungal metabolites. Academic, London
75. Schlingmann G et al (2007) J Nat Prod 70:1180–1187
76. Henrikson JC, Hoover AR, Joyner PM, Cichewicz RH (2009) Org Biomol Chem 7:435–438
77. Bugni TS, Abbanat D, Bernan VS, Maisese WM, Greenstein M, Van Wagoner RM, Ireland CM (2000) J Org Chem 65:7195–7200
78. Ates S, Ozenir S, Gokdere M (2006) Appl Biochem Microbiol 42:500–501
79. Rao KCS, Divakar S, Babu KN, Rao AGA (2003) J Antibiot 55:789–793
80. Sørensen JL, Phipps RK, Nielsen KF, Schroers HJ, Frank J, Thrane U (2009) J Agric Food Chem 57:1632–1639
81. O'Brien M, Nielsen KF, O'Kiely P, Forristal PD, Fuller H, Frisvad JC (2006) J Agric Food Chem 54:9268–9276
82. Herebian D, Zuhlke S, Lamshoft M, Spiteller M (2009) J Sep Sci 32:939–948
83. Zhang YP, Zhu TJ, Fang YC, Liu HB, Gu QQ, Zhu WM (2007) J Antibiot 60:153–157
84. Lang G et al (2008) J Nat Prod 71:1595–1599
85. Burton IW, Quilliam MA, Walter JA (2005) Anal Chem 77:3123–3131
86. Kersters K, Deley J (1963) Biochim Biophys Acta 71:311
87. Challenger F, Subramaniam V, Walker TK (1927) J Chem Soc 200–208
88. Turner WB, Aldridge DC (1983) Fungal metabolites II. Academic, London
89. Rosenberj AJ, Nisman B (1949) Biochim Biophys Acta 3:348–357
90. Wehmer C (1918) Ber Dtsch Chem Ges 51:1663–1668
91. Nair MG, Burke BA (1988) Phytochemistry 27:3169–3173
92. Yu J, Tamura G, Takahash N, Arima K (1967) Agric Biol Chem 31:831–836
93. Alvi KA, Nair BG, Rabenstein J, Davis G, Baker DD (2000) J Antibiot 53:110–113
94. Caesar F, Jansson K, Mutschle E (1969) Pharm Acta Helv 44:676–680
95. Curie JN (1917) J Biol Chem 31:15–37
96. Ye YH, Zhu HL, Song YC, Liu JY, Tan RX (2005) J Nat Prod 68:1106–1108
97. Kimura Y, Baba K, Hata K (1983) Planta Med 48:164–168
98. Almassi F, Ghisalberti EL, Rowland CY (1994) J Nat Prod 57:833–836
99. Isogai A, Washizu M, Kondo K, Murakoshi S, Suzuki A (1984) Agric Biol Chem 48:2607–2609
100. Fukuda T, Hasegawa Y, Hagimori K, Yamaguchi Y, Masuma R, Tomoda H, Omura S (2006) J Antibiot 59:480–485
101. Weidenmueller H-L, Cavagna F, Fehlhaber H-W, Praeve P (1972) Tetrahedron Lett 33:3519–3522
102. Hasegawa Y, Fukuda T, Hagimori K, Tomoda H, Omura S (2007) Chem Pharm Bull 55:1338–1341
103. Fukami H (1991) Patent JP3118376-A
104. Fujimoto Y, Miyagawa H, Tsurushima T, Irie H, Okamura K, Ueno T (1993) Biosci Biotechnol Biochem 57:1222–1224
105. Sakurai M, Kohno J, Yamamoto K, Okuda T, Nishio M, Kawano K, Ohnuki T (2002) J Antibiot 55:685–692
106. Gorst-Allman CP, Steyn PS, Rabie CJ (1980) J Chem Soc Perkin Trans I 2474-2479
107. Rabache M, Neumann J, Lavollay J (1974) Phytochemistry 1974:637–642
108. Tanaka H, Wang P-L, Yamada O, Tamura H (1966) Agric Biol Chem 30:107–113
109. Wang PL, Tanaka H (1966) Agric Biol Chem 30:683–687
110. Galmarini OL, Stodola FH (1965) J Org Chem 30:112–115
111. Zhang Y, Li XM, Wang BG (2007) J Antibiot 60:204–210
112. Ehrlich KC, DeLucca AJ, Ciegler A (1984) Appl Environ Microbiol 48:1–4
113. Guang-yi L, Lenz J, Franck B (1989) Heterocycles 28:899–904
114. Alfatafta AA, Dowd PF, Gloer JB, Wicklow DT (1996) US Patent 5(519):052
115. Ui H et al (2001) J Antibiot 54:234–238
116. Tepaske MR, Gloer JB, Wicklow DT, Dowd PF (1989) J Org Chem 54:4743–4746
117. Tepaske MR, Gloer JB, Wicklow DT, Dowd PF (1989) Tetrahedron Lett 30:5965–5968
118. Varga J, Kevei F, Hamari Z, Toth B, Teren J, Croft JH, Kozakiewicz Z (2000) In: Samson R, Pitt JI (eds) Integration of modern taxonomic methods from *Penicillium* and *Aspergillus* classification. Harwood, Amsterdam, pp 397–411
119. Sings HL, Harris GH, Dombrowski AW (2001) J Nat Prod 64:836–838
120. Tepaske MR, Gloer JB, Wicklow DT, Dowd PF (1989) Tetrahedron 45:4961–4968
121. Kodukula K, Arcuri M, Cutrone JQ, Hugill RM, Lowe SE, Pirnik DM, Shu Y-Z (1995) J Antibiot 48:1055–1059
122. Tepaske MR, Gloer JB, Wicklow DT, Dowd PF (1991) Tetrahedron Lett 32:5687–5690
123. Kobbe B, Cushman M, Wogan GN, Demain AL (1977) Appl Environ Microbiol 33:996–997
124. Akiyama K, Teraguchi S, Hamasaki Y, Mori M, Tatsumi K, Ohnishi K, Hayashi H (2003) J Nat Prod 66:136–139
125. Ikeda S, Sugita M, Yoshimura A, Sumizawa T, Douzono H, Nagata Y, Akiyama S (1990) Int J Cancer 45:508–513
126. Priestap HA (1984) Tetrahedron 40:3617–3624
127. Umezawa H, Tobe H, Shibamoto N, Nakamura F, Nakamura K, Matsuzaki M, Takeuchi T (1975) J Antibiot 28:947–952
128. Tobe H, Naganawa H, Takita T, Takeuchi T, Umezawa H (1976) J Antibiot 29:623–625
129. Savard ME, Miller JD, Blais LA, Seifert KA, Samson RA (1994) Mycopathologia 127:19–27

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